DOCTOR OF PHILOSOPHY

Exploiting crosstalk between growth and immunity in plants the role of the Phytophthora infestans effector AVR2 in potato late blight

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Exploiting crosstalk between growth and immunity in plants: the role of the *Phytophthora infestans* effector AVR2 in potato late blight

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

At	*Arabidopsis thaliana*
AVR	avirulence
BL	brassinolide
BLAST	basic local alignment search tool
BR	brassinosteroid
Bp	base pairs
CBEL	cellulose-binding elicitor lectin
CRN	crinkling and necrosis
Dpi	days post infiltration/inoculation
ET	ethylene
GFP	green fluorescent protein
EBL	epibrassinolide
ETI	effector-triggered immunity
ETS	effector-triggered susceptibility
hpi	hours post infiltration/inoculation
HR	hypersensitive resposne
JA	jasmonic acid
kDa	kilodaltons
LRR-RLK	leucine-rich-repeat receptor-like kinase
MAPK	mitogen-activated-protein-kinase
MAMP/PAMP	microbe/pathogen associated molecular pattern
Ml	millilitres
Mb	megabases
Nb	*Nicotiana benthamiana*
NB-LRR	nucelotide binding leucine-rich repeat
OD	optical density
Pi	*Phytophthora infestans*
PR	pathogenesis related
PCD	programmed cell death
PCR	polymerase chain reaction
PTI	PAMP or Pattern-Triggered-Immunity
QTL	quantitative trait loci
QRL	quantitative resistance loci
RBBH	Reciprocal best BLAST hit
ROS	reactive oxygen species
R	resistance
RxLR-EER	arginine - any amino acid – leucine – arginine – glutamate – glutamate - arginine
rpm	revolutions per minute
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
SA	salicylic acid
SAR	systemic acquired immunity
SEM	standard error of the mean
St	*Solanum tuberosum*
Sd	*Solanum demissum*

T3SS	Type 3 secretion system
VIGS	virus induced gene silencing
Y2H	Yeast-2-Hybrid
µg	micrograms
µl	microlitres

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Declaration

I declare that I am the author of this thesis, and the research described in this work is my own.

All references cited in this work have been consulted by myself, and contributions from others are clearly identified and acknowledged.

This work has not been previously accepted for any other higher degree.

Dionne Turnbull

We certify that Dionne Turnbull has fulfilled the relevant Ordinance and Regulations of the University Court and is qualified to submit this thesis for the degree of Doctor of Philosophy.

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Abstract

Plants are fundamental to life on earth, crucially providing the basis of our food supply. As world population continues to grow, so too does the pressure on our agricultural systems, with one of the biggest challenges being the control of plant pathogens to ensure a healthy crop.

The interaction between plant and pathogen is complex, with subtleties at the molecular level dictating the boundary between health and disease. This is exemplified by pathogen effectors; secreted proteins which enter the plant cell and interact with host targets to facilitate infection. AVR2 is one such effector, secreted by the oomycete pathogen *Phytophthora infestans*; the pathogen responsible for potato late blight. AVR2 interacts with a family of kelch-repeat containing phosphatases in potato, the BSLs, implicated in brassinosteroid pathway signalling – a major hormone signalling pathway in plants associated with growth and development. This work investigates the role of AVR2 and its host targets in pathogen virulence, with focus on the link between the brassinosteroid pathway and immunity in *S. tuberosum*.

StBSL1 is shown to be a 'susceptibility factor' in *P. infestans* infection – a host protein with a positive effect on pathogen virulence. AVR2 stabilises BSL1 *in planta*, and both AVR2 and BSL1 are shown to suppress primary defence responses in the plant.

Transcriptional analysis of brassinosteroid-treated *S. tuberosum* is used to identify a set of marker genes for active BR signalling. Strikingly, AVR2 is shown to upregulate

this pathway, and specifically upregulates the transcription factor StHBI1-like, identified as a suppressor of immunity.

These findings reveal a novel mechanism in oomycete effector biology; the exploitation of crosstalk between the brassinosteroid pathway and immune signalling in plants.

CHAPTER 1

Introduction

No living thing exists in isolation; each is surrounded by a plethora of other organisms, and dynamic environmental conditions. Interactions occur at many levels, with signals generated, perceived, and integrated, enabling the organism to respond in a manner relevant to the conditions present. This is exemplified by the interaction between a pathogen and host, which dictates the boundary between health and disease.

Plants are fundamental to life on earth, as producers of oxygen, medicines, fuel and other valuable materials, as well as playing a key role in the water cycle and climate. Crucially, they are the cornerstone of our food supply. Whether directly or indirectly, everything that we eat originates in plants, thus we depend on their health to ensure our own. Crop diseases threaten the security of our food supply, with those caused by micro-organisms such as bacteria, fungi, viruses, or oomycetes claiming 10-16% of the total global harvest (Chakraborty and Newton, 2011). Particularly in the developing world, many small-holders rely on their crop for basic sustenance, and a disease outbreak can be devastating. In the developed world, access to ever-improving knowledge and technology means that disease outbreaks in crops are no longer a matter of life or death, but nonetheless, the financial consequences for both the farmer and the wider economy can be significant.

1.1 *Phytophthora infestans* and potato late blight

Arguably the single most notorious crop disease in the history of agriculture, potato late blight, is caused by the oomycete *Phytophthora infestans*, translated as 'the infesting plant destroyer'. Late blight decimated the potato harvest in Ireland in the mid-1840s. This was known as the Great Famine, and resulted in the loss of almost one million lives and mass emigration of many more from the country (Birch and Cooke, 2013). The disease became pandemic, and was also a major contributing factor in the Continental Famine of mainland Europe (Delanghe *et al*., 2013). Loss of the potato crop, combined with poor wheat and rye harvests during the same period, led to massive loss of life in addition to precipitating radical changes in economy and politics (Vanhaute *et al*., 2006).

Potato (*Solanum tuberosum*) is currently the number one non-cereal crop in global food production. Although potato production in developed countries is beginning to decline, cultivation is booming in the developing world, increasing by 5% annually (Prakesh, 2008). With higher yield per hectare than cereals (Lokossou, 2009), and high nutritional value in the form of its starchy tubers, potato remains a valuable staple in the diet of many worldwide. *Phytophthora infestans* continues to be a serious threat to potato production – disease can spread field-wide within a matter of days, with yield losses up to 100%, and incurring costs in excess of £5 billion worldwide (Havervort *et al*., 2008). Notably, disease control accounts for a significant part of the carbon footprint of potato production, estimated at 10% (Harverkort and Hillier, 2011).

Fungicides remain the mainstay of the farmer's defence against potato late blight, reflected in the Potato Council (2013) advice; "If you can't spray it, don't plant it".

Multiple preventative sprays per season are required – as often as weekly during peak blight conditions. This situation is far from ideal, with environmental costs not only directly from the chemicals themselves, but also from the increased energy inputs required to produce and apply them. With an EU directive (Directive 2009/128/EC) driving a move toward more sustainable use of pesticides, the future may see many of the key chemical controls for blight either banned or severely limited. Blight control presents an even bigger challenge for the organic farmer, who cannot use synthetic pesticides. Instead, they are limited to organic alternatives such as copper sulphate; shown to be of significantly higher toxicity, and much more persistent in the environment, than the most common synthetic option Mancozeb (Trewavas, 2004).

Field management practices such as crop rotation and waste pile management also play a part in preventing the disease, and systems are in place for the monitoring of blight outbreaks (Fight Against Blight, AHDB 2015), and the prediction of high risk weather conditions for blight, known as Smith Periods (Blightwatch, Met Office 2016).

1.2 Host Breeding and Resistance

Attempts to breed resistant varieties have had limited success, with the pathogen displaying an impressive ability to overcome selection pressures. Early breeding efforts focused on the introgression of 11 resistance genes identified in the wild relative *Solanum demissum* which, although were initially successful, were soon overcome by newly evolving pathogen isolates capable of evading detection (Vleeshouwers, 2011). Even deploying *R*-genes in combination cannot guarantee durability. Fry (2008) gives Pentland Dell as an example – a Scottish cultivar bred to contain a pyramid of three R

genes (R1, R2 and R3) to provide blight resistance. Within four years of commercial production, this had been overcome by evolving pathogen populations. McDonald and Linde (2002) describe the 'boom and bust' cycles that frequently occur with *R* gene deployment, with great success followed quickly by resistance breakdown, attributed to the extreme selection pressure put upon the pathogen.

Hein *et al*. (2009) describe *R* gene 'hot spots'; clusters of resistance genes that are more than the sum of their apparent parts. It is acknowledged that genes in close proximity to these *R* gene clusters may well play a role in 'quantitative' or 'field' resistance - that is, genes which do not provide full resistance alone, but contribute small effects, as opposed to the full or 'qualitative' resistance that R gene deployment can provide. Quantitative resistance is thought to offer more broad-spectrum disease resistance, and the multiple genes involved may make the resistance more durable (Rauscher *et al*., 2010). One example of this is potato allene oxide synthase 2 (StAOS2), a cytochrome p450 enzyme involved in the synthesis of jasmonates required in defence signalling (Pajerowska-Mukhtar *et al*., 2008). This gene co-localises with *R* genes on chromosome XI of potato, and is associated with increased resistance to both late blight and infection by *Pectobacterium atrosepticum*, the pathogen responsible for black leg and tuber soft rot. Despite much research into quantitative resistance, a strong link between foliage resistance and late maturity of the crop (Bradshaw *et al*., 2004) presents a challenge for its application.

It may be that gene pyramiding with both elements of quantitative and qualitative resistance offers an option with higher durability. Brun *et al.* (2010) demonstrate the potential of this approach by comparing the durability of the resistance gene *RLM6* in

two cultivars of *Brassica napus*; one of which has several quantitative trait loci (QTL) that convey higher quantitative resistance against the blackleg fungus *Leptosphaeria maculans*. Whilst the *R* gene alone was overcome within three seasons, the *R* gene in combination with QTLs remained effective at the end of the five-year trial.

Regardless of the resistance strategy, the tetraploid genome of *Solanum tuberosum* makes traditional breeding methods lengthy and difficult. Havervort *et al*. (2008) estimate that genetic modification could save 15 years or more in the development of new cultivars. The authors note that these could be 'cisgenic' as opposed to 'transgenic', whereby the genes are from a related wild species that could in fact have been bred in the traditional manner. This may present a form of biotechnology deemed more acceptable by those who currently oppose its use. Strategies for breeding resistance are discussed further in **Chapter 6**.

1.3 Biology of the Pathogen

Phytophthora infestans is an oomycete; a filamentous eukaryote. Classified as fungi until the end of the 20th century (Fry, 2008), oomycetes are now considered a distinct lineage in the kingdom Chromalveolata. The genus *Phytophthora* contains several high profile pathogens, including *P. ramorum* (the cause of Sudden Oak Death), *P. capsici* (affecting Cucurbits among others) and *P. sojae* (affecting soybean). *P. infestans'* main host is potato, but it can also infect tomato (*Solanum lycopersicon*) and several other Solanaceous species such as the model plant *Nicotiana benthamiana*. Late blight is noted as the first plant disease for which a micro-organism was shown to be the cause,

thus the pathogen can be considered as contributing to the birth of plant pathology as a field of research (Schumann and D'Arcy, 2000).

Asexual reproduction is the most common life habit of *P. infestans*, beginning with the production of sporangia, which may germinate directly at higher temperatures (20 - 27°C), or produce motile biflagellate zoospores at lower temperatures (4 - 12°C). Sporangia are dispersed by air currents and water droplets, deposited elsewhere on the same plant or further afield. Zoospores can emerge within two hours in cool conditions, requiring water for motility, and encyst on the plant leaf surface before germinating to initiate new infection. Inoculum can also be washed into the soil, leading to the infection of tubers. The life cycle can be completed in only a matter of days, with a new generation of sporangia formed and released - up to 300,000 per lesion (Fry, 2008). The life-cycle of *P. infestans* is represented in **Figure 1.1**.

Infection of plant material involves the development of a germ tube from the cyst or sporangia, the tip of which swells to become an appressorium and subsequently a penetration hypha that breaches the cuticle and cell wall (Avrova *et al*., 2008). These produce finger-like haustoria that invaginate host cells, in intimate association with the cell membrane (**Figure 1.2**). Signalling and nutrient acquisition occurs via this association, with proteins and other small molecules secreted by the pathogen, termed 'effectors'. Effectors function to promote pathogenicity, for example by modulating the plant immune response, or manipulating host processes to the pathogens benefit. Effectors are described in detail in **Section 1.4.2**.

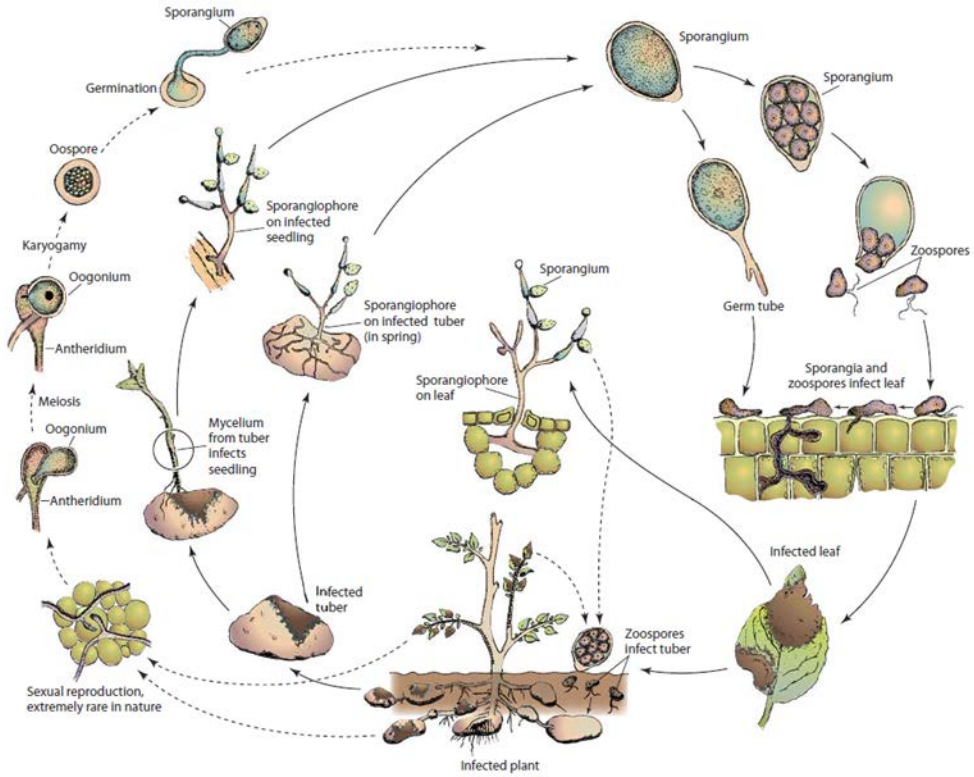

Figure 1.1 The life cycle of *Phytophthora infestans*

The oomycete responsible for potato late blight displays a complex life-cycle, capable of both sexual and asexual reproduction. Fruiting bodies produce sporangia, which may germinate directly, or rupture to release zoospores depending on environmental conditions. Reproduced from Agrios (2005).

Interaction between *P. infestans* and the host plant is biotrophic in the first instance, with infection transitioning to a necrotrophic phase after approximately 36 hours, during which haustoria are no longer formed (Avrova, 2008). The molecular mechanisms that govern the transition between biotrophy and necrotrophy remain poorly understood, but have been shown to involve distinct patterns of gene expression; the switch correlates with a decrease in effector gene expression, and an increase in necrosis-inducing gene expression (Pais *et al*., 2013). Kelley *et al*. (2010) describe the identification of PiSNE1- a secreted effector expressed during the biotrophic phase, capable of suppressing host cell death. This is in contrast to the

activity of necrosis-inducing peptides, such as PiNPP1.1, shown to accumulate in the later stages of infection (Kanneganti *et al.*, 2006). Lee and Rose (2010) show that SNE1 can directly suppress the cell death triggered by NPP1.1, and propose a conceptual model where this antagonism can regulate the switch between the two pathogen lifestyles.

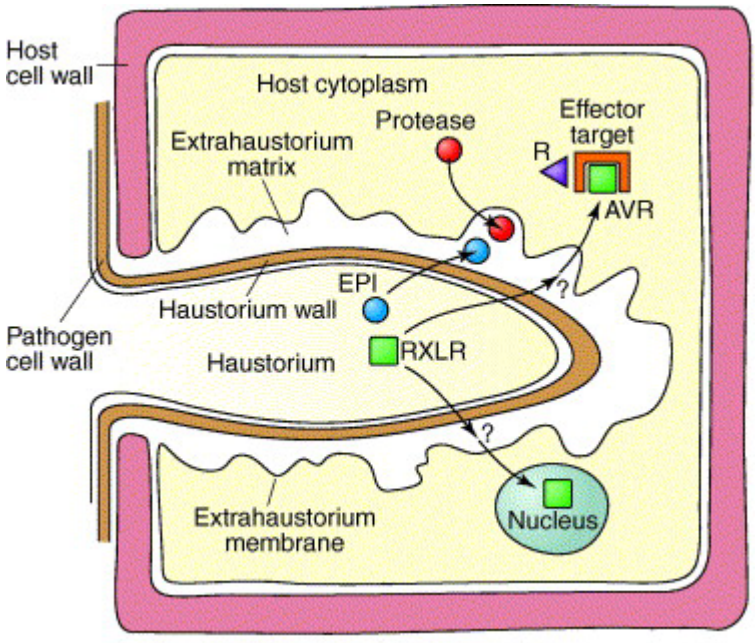

Figure 1.2 *Phytophthora infestans* haustoria and effector secretion

P. infestans forms haustoria, finger-like protrusions that invaginate plant host cells. Effector proteins are secreted from these structures, and are translocated into the plant cell where they may act to suppress immunity or modify other host processes to support pathogenicity. Reproduced from Birch *et al*. (2006).

Two mating types of *P. infestans* exist; A1 and A2, and sexual reproduction can occur when these co-exist in the host plant. Mating types are bisexual and may form male or female structures (antheridia or oogonia respectively) from which nuclei fuse and form a diploid oospore. These are thick-walled and may survive in soil for years, providing

long-term inoculum in fields that standard crop-rotation practices may not deal with effectively. Turkensteen (2000) notes variability in oospore production between infections of various potato cultivars, partly attributed to the level of disease resistance (medium resistance correlates with more oospores) and potentially linked to varying sterol content.

Sexual reproduction of *P. infestans* was not a widespread issue in Europe until relatively recently, with the population consisting of A1 genotypes only. The A2 type was first detected in Europe in 1984 (Goodwin and Drenth, 1997). A2 was previously confined to populations in Mexico; the centre of *P. infestans* origin and a site of remarkable genetic diversity for both the pathogen and wild *Solanum* species. The A2 mating type reached a level of dominance in the UK in 2007, with genotype 13_A2 (known as 'Blue 13') overtaking other previously dominant lineages. Blue 13 has high resistance to phenylamide fungicides (Gisi *et al*., 2011), and has overcome the blight resistance of the potato cultivar 'Stirling'. Populations are dynamic, changing rapidly, and in 2011 Blue 13 was overtaken by an A1 genotype referred to as 'Pink 6' (Cooke *et al*., 2012). The increase of novel genotypes, often unique to one site in a single year, is also noted; suspected evidence of sexual recombination in the field. However, despite the presence of both mating types, differences in ploidy mean that sexual recombination is not always possible. Li *et al*. (2015) show sexuality to be largely limited to diploid lineages, and show that the asexual lineages (including the highly aggressive 13_A2) are largely triploid. These authors postulate that polyploidy has played a role in the success of these clonal lines, as it can effectively buffer deleterious mutations that can accumulate in asexual reproduction.

The genome of *Phytophthora infestans* has now been sequenced, providing insight into how the pathogen functions. At a relatively large 240Mb, Haas *et al.* (2009) report the unusual genome format; with repeat-rich blocks of low gene density interspersed throughout. 74% of the genome consists of repeats, with more transposable elements than any genome described to date. The effector gene complement of *P. infestans* is largely found in these repeat-rich areas, with these features thought to play a major role in the pathogens adaptability, allowing rapid evolution and expansion of populations.

1.4 The molecular basis of disease versus resistance

Plants are capable of intricate immune responses, resulting in resistance to the majority of potential pathogens. However, in modern agricultural settings that often consist of huge areas of crops with little genetic variation, the relatively few microbes that can infect can cause damage on a massive scale. Passive protection measures are the first line of defence, with the leaf waxy cuticle, plant cell wall, and secreted antimicrobials providing non-host resistance (Mysore *et al*., 2004). Beyond this, interactions become increasingly complex and specific, and represent generations of co-evolution between plant and microbe. Jones and Dangl (2006) represent innate immunity in plants as a 'zig-zag' model (see **Figure 1.3**). This provides an evolutionary framework for plant-microbe interactions of a biotrophic nature, involving several phases which will be discussed in turn.

1.4.1 Pattern-triggered immunity (PTI)

For micro-organisms that can breach the plants constitutive defences, conserved molecular signatures known as microbe or pathogen-associated-molecular-patterns (MAMPs or PAMPs) can alert the plant to their presence, and lead to pattern-triggered immunity (PTI). PAMPs are often small regions of proteins, peptides, which are highly conserved among a class of micro-organisms. Required for pathogen fitness, they are evolutionarily stable and therefore provide a durable means of recognising 'non-self' for the plant. Additionally, PAMPs are considered to act only outside the plant cell membrane (Gijzen and Nurnberger, 2006).

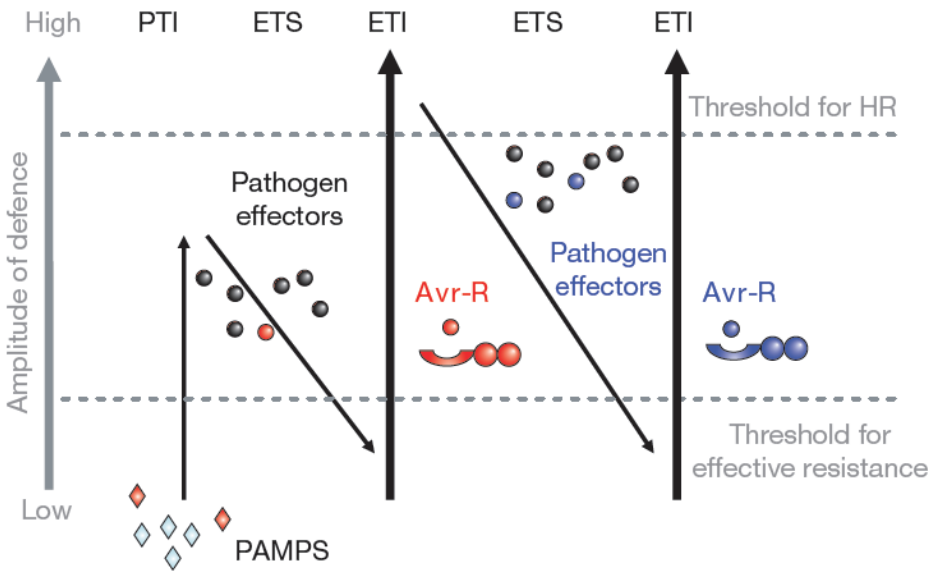

Figure 1.3 The 'zig-zag' model of plant pathogen interactions

This model provides an evolutionary framework for biotrophic plant-microbe interactions. PAMPs may be recognised by plant PRRs, triggering PTI. Some pathogens may secrete effectors to overcome this immune response, leading to effector triggered susceptibility (ETS), but these may be recognised by plant R-proteins, leading to effector-triggered immunity (ETI). Subsequent phases of ETS and ETI can occur by means of the molecular 'arms-race' that puts strong selection pressure on the pathogen to evade recognition, and on the plant to improve recognition. Reproduced from Jones and Dangl (2006).

These PAMPs are recognised by transmembrane plant proteins referred to as pattern recognition receptors (PRRs), and successful detection triggers a wide range of physiological responses such as the production of reactive oxygen species (ROS), cell wall reinforcement by means of callose deposition, activation of mitogen-activated protein kinases (MAPKs), and major changes in gene expression (Zipfel, 2008). The plant hormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) also play a major role in the plant immune response (Ingle *et al*., 2006). Salicylic acid signalling is generally associated with response to biotrophic and hemibiotrophic pathogens, whilst ethylene and jasmonic acid are required for response to necrotrophs and herbivorous insects (Bari and Jones, 2008).

One of the best characterised PAMPs to date is the flg22 peptide from flagellin – an essential building block of bacterial flagella (Felix *et al*., 1999). Chinchilla *et al*. (2006) show the Arabidopsis protein FLS2 (a leucine-rich-repeat receptor-like kinase, or LRR-RLK) to be directly responsible for flg22 perception. Notably, this PRR is analogous to the toll-like receptor TLR5, highly conserved in vertebrates, which functions in flagellin perception and activation of the subsequent innate immune response (Yoon *et al*., 2012).

Several PAMPs have been identified in oomycetes. Brunner *et al.* (2002) show that pep-13, a surface exposed peptide of a cell-wall transglutaminase GP42, elicits PTI in parsley and potato. This peptide appears to be conserved throughout the *Phytophthora* genus, with the functional protein involved in protein cross-linking and tissue regeneration (Langston, 2007). Also located in the pathogen cell wall is a cellulose-binding elicitor lectin (CBEL).This was cloned from *P. parasitica* by Mateos *et*

al. (1997), and shown to elicit necrosis and the up-regulation of defence genes. The CBEL domain is present in a number of *Phytophthora* species and has been suggested to be conserved throughout the oomycetes (Torto-Alilibo *et al*., 2005). The gene has been implicated in developmental processes in *Phytophthora*, required for cell wall deposition, adhesion, and cellulose perception (Gaulin *et al*., 2002). However, the authors saw only a modest decrease in fitness when mutated, indicating it may be non-essential.

PAMPs are not only structural proteins – some may be secreted. Elicitins are small, highly conserved proteins secreted by *Phytophthora* and *Pythium* species. Known to trigger defence responses in plants, some have been shown to bind sterol and function as extracellular sterol carriers (Mikes *et al*., 1998). *P. infestans*, like other oomycetes in the order Peronosporales, cannot synthesise its own sterols and must acquire them from the host plant (Gaulin *et al*., 2010). INFESTIN1 (INF1) is one such elicitin, shown in tomato to generate ROS bursts, activate JA and ET mediated signalling pathways, and induce resistance to bacterial wilt (Kawamura *et al*., 2008). The receptor-like protein ELR, from the wild Solanaceous species *S. microdontum,* has been recently identified as mediating INF1 recognition (Du *et al*., 2015). This protein conferred increased resistance to *P. infestans* when introduced into cultivated *S. tuberosum* cv. Desiree. In addition, ELR can also perceive several other elicitins from a range of *Phytophthora* species, so its introgression presents a potential route for developing increased disease resistance.

Some PAMPs span more than a single class of micro-organism. The necrosis and ethylene inducing peptide1 (NEP1)-like peptides (NLPs) are found in fungi and bacteria

as well as oomycetes (Oome *et al*., 2014). These are cytotoxic, and have recently been shown to elicit plant immune responses in two ways; both by typical PAMP perception of a specific peptide, and by causing cell damage resulting in the production of DAMPs (damage-associated molecular patterns), which can be recognised in a similar manner to PAMPs (Bohm *et al*., 2014).

1.4.2 Pathogen effector proteins

Biotrophy as a life style is hazardous – imposing on a living host runs the risk of detection. To counteract the plant defences induced by PAMP perception, pathogens have evolved sophisticated means of suppressing, evading, or manipulating these responses to their benefit, by means of 'effector proteins', also described as virulence factors. Effectors may be deemed avirulence (AVR) proteins if recognised by a corresponding resistance gene in the plant, or they may go undetected, contributing to successful colonisation and disease. While PAMPs are defined as being conserved throughout microbial classes, and contributing to general fitness, effectors are defined as species or race specific, contributing to virulence (Chisholm *et al*., 2006).

Plants secrete hydrolytic enzymes, such as chitinases, proteases and glucanases, as part of their defence repertoire (Hein *et al*., 2009). Many pathogens have adapted to overcome this; for example the fungus *Cladosporium fulvum* secretes the effector Avr4 to suppress the activity of tomato plant chitinases (Van den Berg *et al*., 2006). Unlike fungi, the oomycete cell wall contains little or no chitin, so chitinases are not an issue, but they do require a counter-attack against glucanases and proteases. Rose *et al*. (2002) report the identification of a glucanase inhibiting protein, GIP1, that interacts directly with the soybean endoglucanse EGaseA, rendering it ineffective. GIP1 was

identified in *P. sojae*, a soybean root pathogen, and 4 homologues of the gene have been identified in *P. infestans* (Hein *et al*., 2009).

During infection of tomato, *P. infestans* has been shown to upregulate EPI1, a kazal-like serine protease inhibitor (Tian *et al*., 2004). EPI1 was found to directly target and inhibit the tomato subtilisin-like serine protease P69B. The authors note that kazal-like protease inhibitors are found in apicomplexan parasites, eg. *Plasmodium* species, indicating potentially conserved virulence strategies across a broad range of pathogenic organisms.

The above examples are extracellular effectors, with interaction and outcome occuring outside the plant host cell membrane. Other effectors work intracellularly, and in some cases act to directly supress PTI. Host cell targeting of effectors is achieved via the type III secretion system (T3SS) in bacteria; a well-characterised structure allowing direct 'injection' of effector proteins into the host cytosol (Buttner and He, 2009). Two such effectors are AvrPto and AvrPtoB, secreted by the bacterial pathogen *Pseudomonas syringae*. AvrPto and AvrPtoB both bind to the kinase BAK1, shown to prevent its association with FLS2, thus inhibiting PTI signalling (Shan *et al.*, 2008).

Unlike the T3SS for effector delivery in bacterial pathogens, the mode of entry for oomycete effectors is less well understood. Rehmany *et al*. (2005) describe the discovery of a common motif in effector proteins from the downy mildew *Hyaloperonospora parasitica*, *P. infestans*, and *P. sojae*, later shown to be present in many diverse secreted oomycete proteins. The 'RXLR' motif (arginine-any amino acid-leucine-arginine) is found within 32 amino acids of the signal peptide, and is commonly followed by the sequence 'EER'. This motif shows similarity to translocation signals in

Plasmodium species, and it was hypothesised that it could be involved in trafficking proteins into the host.

Whisson *et al.* (2007) proved this theory to be correct, showing that the *P. infestans* effector Avr3a could not be translocated into the host without the RXLR-EER motif. The protein was targeted to the haustoria and secreted into the extrahaustorial matrix both with and without the RXLR. The expression levels of multiple *P. infestans* RXLR-EER class proteins were also studied, shown to be induced and upregulated during infection of potato, characteristic of pathogen effectors.

To date, several RXLR effectors have been termed avirulence proteins (AVR) on the basis of their recognition by a corresponding resistance gene. All RXLR effectors have a modular structure consisting of a signal peptide for secretion, the conserved RXLR domain for translocation, and a variable C-terminus responsible for effector function. The RXLR domain has been shown to be over-represented in pathogenic oomycete genomes (Win *et al*., 2007), and Birch *et al*. (2008) draw attention to the fact that it has not yet been targeted for recognition by the host plant. The authors note the significant presence of the RXLR motif in the Arabidopsis proteome, with many of these proteins potentially involved in endocytosis. It may be that the pathogen has evolved to exploit host endocytosis for effector delivery (Birch *et al*, 2008) and as the RXLR domain exists in host as well as pathogen, it is not a suitable candidate for recognition.

Currently, there are around 563 RXLRs predicted in the *P. infestans* genome (Haas, 2009), indicating a wealth of information on potential host-pathogen interaction still to be discovered. Research to date has identified Avr3a (Armstrong *et al*., 2005), AVR-

blb1 (Vleeshouwers, 2008), AVR4 (van Poppel, 2008), AVR-blb2 (Oh *et al*., 2009) and AVR2 (Gilroy *et al*., 2011) as avirulence proteins, among others.

Bos *et al*. (2010) showed that the effector PiAVR3a suppresses recognition of the secreted PAMP INF1. Yeast-2-hybrid analysis revealed PiAVR3a to interact with CMPG1, an E3 ligase required for INF1-mediated cell death. While usually degraded by the proteasome during PCD, PiAvr3a stabilises CMPG1 and prevents its action. Additionally, PiAVR3a was also shown to interact with the exocyst components SEC3 and SEC5, implicated in the endocytic cycle. The authors showed PiAVR3a to be an essential effector, as it compromises pathogenicity when silenced. A similar virulence strategy has been identified for PiAVR1; shown to also interact with SEC5, and to suppress callose deposition (Du *et al*., 2015).

Another class of oomycete effectors, the 'Crinklers', or CRNs (crinkling and necrosis) have been identified by Torto *et al.* (2003). Shown to be translocated into the host (Schornack *et al*., 2010) and targeted to the nucleus (Stam *et al*., 2013), their function in virulence is as yet uncharacterised.

If effector proteins are successful in suppressing host immunity, or in their manipulation of plant processes, the plant is described as being in a state of effector-triggered susceptibility (ETS) and disease may result. However, co-evolution has fostered the recognition of some of these effectors, which can trigger further immune response by the plant; termed effector-triggered immunity or ETI.

1.4.3 Resistance Genes and ETI

Flor devised the gene-for gene concept in 1942, observing segregation in the resistance of flax to the rust fungus *Melampsora lini*. He theorised that for every gene involved in disease response in the host, there was a corresponding pathogenicity gene in the pathogen. This concept forms the basis of effector-triggered immunity by plant resistance (R) proteins, although complexities have been revealed over time (Gassmann and Bhattacharjee, 2012). As well as gene-for-gene, there are also cases of genes-for-gene (where multiple effectors can be recognised by a single *R* gene) and gene-for-genes (where a single effector is recognised by a combination of *R* genes). Additionally, some effectors such as AVR3a (Bos *et al*., 2010) and AVR2 (Gilroy *et al*., 2011) have been shown to exist as multiple variants which may or may not be recognised, so the term 'allele-for-allele' may be more appropriate.

ETI is generally considered to be a faster, stronger version of PTI, with overlapping signalling outputs including oxidative bursts, MAPK activation, and hormonal changes, although these responses may be more prolonged in ETI (Tsuda and Katagiri, 2010; Ingle, 2006). In addition, ETI is more frequently associated with the hypersensitive response or HR; a form of programmed cell death localised to the site of infection (Heath, 2000). The HR is generally considered to function in preventing the spread of biotrophic infection, by starving the imposing pathogen of living tissue. However in some cases, disease resistance can be separated from the HR, indicating that the cell death itself may not always be an essential part of defence, and may be a consequence of signalling, or function in signalling itself (Richael and Gilchrist, 1999; Heath, 2000). Notably, whilst the HR can be part of an effective defence against biotrophic pathogens, the opposite is true for a necrotrophic pathogen. Dead tissue will serve to

assist infection, and the hypersensitive response can be exploited by necrotrophic pathogens such as *Botrytis cinerea* (Govrin and Levine, 2010).

R genes, which facilitate effector recognition, largely encode NB-LRR proteins – with nucleotide binding (NB) domains, and a variable number of leucine-rich repeats (LRRs). These represent one of the largest gene families in plants, with 438 NB-LRRs in the genome of *S. tuberosum* Group Phureja DM (Jupe *et al*., 2012). NB-LRRs can be broadly grouped into two classes; the CC-NB-LRRs, with a coiled-coil domain at the N-terminus, and the TIR-NB-LRRs, with a Toll/interleukin-1 receptor-like domain at the N-terminus, bearing similarity to mammalian immune receptors. The modular structure of these proteins enables their function not only as sensors but as switches, with the interaction between domains proposed to control the on/off state of the protein (Takken and Goverse, 2014).

Interactions between pathogen effectors and plant host resistance proteins may be direct or indirect. Given the fact that a single plant may be faced with the effector complement of several pathogens, direct recognition by means of a separate resistance protein for each effector would require huge amounts of resources. This may be why examples of direct recognition are much less common (Birch *et al.,* 2006). Indirect recognition may offer *R* genes the capacity to detect multiple effectors, potentially targeting 'hubs' that represent key aspects of host defence.

Direct recognition of an effector by a NB-LRR resistance protein is seen in the case of Rpi-BLB1 (also called RB) and effector AVR-BLB1 (also called IPI0). Song *et al.* (2003) describe the discovery of Rpi-blb1 from the wild Solanaceous species *Solanum bulbocastanum*. Thought to confer broad-spectrum resistance to all known races of *P.*

infestans, it emerged recently that effector variants exist that can overcome this resistance. Halterman *et al.* (2010) describe the analysis of forty *P. infestans* isolates from geographically diverse locations, revealing that Ipi0 is a multi-gene family, with isolates containing a large number of variants. The variant IPI-04 overcomes resistance by directly binding to RB and preventing detection of the other IPI0 forms. Another example of direct recognition, which exemplifies the 'genes-for-gene' concept, is the rice NB-LRR protein RGA5, capable of direct interaction with both AVR1-C039 and AVR-Pia, two unrelated effectors secreted by the rice blast fungus *Magnaporthe oryzae* (Cesari *et al*., 2013).

To explain the mechanism of indirect interaction between effector and R protein, Van der Biezen and Jones (1998) postulate a 'guard' hypothesis, whereby a pathogen effector targets a specific plant protein (the guardee) which is safeguarded by a particular resistance protein. The best characterised example of this in action is the Arabidopsis protein RIN4, which is targeted by three independent effectors from *P. syringae*; AvrRPM1, AvrB, and AvrRpt2. Modification of RIN4, either by effector-induced phosphorylation or cleavage, activates the NB-LRRs RPM1 or RPS2 respectively (Jones and Dangl, 2006; Mackey *et al*., 2002).

Moving beyond the guard hypothesis, Van der Hoorne and Kamoun (2008) note the potentially unstable position of a guarded target, with evolutionary pressure both to mutate (so as to not be targeted) but also to remain conserved (so it can still be guarded). They propose an extension to the guard hypothesis, termed the 'decoy' model, whereby gene duplications or mimics allow the evolution of a protein solely functioning to detect the effector. One example of a decoy is tomato RCR3, one of two

cysteines proteases inhibited by the *Cladosporum fulvum* effector AVR2, and required for effector recognition mediated by Cf-2 (Dixon *et al*., 2000). This protein does not appear to contribute to pathogen virulence, with evidence suggesting that the other cysteine protease, PIP3, is the true functional target of the pathogen (Shabab *et al*., 2008). Notably, Cf-2 facilitates recognition not only of a fungal effector, but also an example of a plant-parasitic nematode effector; VAP1 from *Globodera rostochiensis*, which interacts with the same plant target RCR3 (Lozano-Torres *et al*., 2012).

Effector-triggered immunity is associated with a plant-wide response known as systemic acquired resistance (SAR) – this primes the plant to defend itself against further attack, and can provide broad spectrum resistance against other pathogens for an extended time. Elevated salicylic acid levels and expression of pathogenesis-related (PR) genes are characteristic of SAR (Durrant & Dong, 2004). Recently, Park *et al*. (2007) showed an inactive SA derivative, methyl salicylate, to be the phloem-mobile signal that triggers SAR in distal plant tissue.

Pathogen effectors can also act to overcome ETI, referred to as ETS2. This 'arms race' can continue, with pathogens evolving new or modified effectors that are not recognised, and plants under selective pressure to evolve new corresponding resistance. It should be noted that plant immunity does not fit perfectly within PTI and ETI – the boundaries can be blurred and considered a continuum (Thomma *et al*., 2011). Additionally, while the zig-zag model (Jones and Dangl, 2006) provides an excellent framework for plant-microbe interactions from an evolutionary perspective, it is limited in that it cannot be used to predict the outcome of a particular plant microbe interaction. Also, it fails to take into account spatial and temporal aspects of

infection. This is particularly relevant in the case of hemibiotrophic infection, where a progressive transition from biotrophy to necrotrophy means interactions can differ vastly even within the same lesion. Additionally, a true plant-microbe interaction involves multiple PAMPs and effectors, multiple PRRs and resistance proteins, and integrates environmental conditions and the developmental state of the plant. A move towards a systems biology approach could allow the development of models which integrate multiple inputs. This would allow prediction of an end result in terms of immune response, exemplified by Pritchard and Birch (2014).

1.5 The AVR2-R2 story

The role of eukaryotic pathogen effectors remains largely unknown; a major limiting factor in our understanding of disease and disease resistance in plants. This work focuses on the *P. infestans* effector AVR2; an RXLR-EER effector which is shown to accumulate at the site of haustoria formation, and is upregulated during biotrophy (Gilroy *et al*. 2011). Breen (2012) identified the host targets of PiAVR2 to be a family of kelch-repeat containing phosphatases; StBSL1, StBSL2a and StBSL2b. These are homologous to the BRI1 SUPPRESSOR1 (BSU1) family members in Arabidopsis, which play a role in brassinosteroid signalling (Mora-Garcia *et al*., 2004). The brassinosteroid pathway is a key hormone signalling pathway involved in plant growth and development, discussed in more detail in **Section 1.6**. A biological rationale for the interaction between PiAVR2 and the StBSL family remained to be determined, and is the focus of this work.

PiAVR2 can be recognised by the resistance protein R2 from *S. demissum.* Shown to be required for AVR2-induced cell death (Vleeshouwers, 2008), *R2* resides at a major late blight locus on linkage group IV that also hosts *Rpi-abpt, Rpi-blb3*, and *R2-like* (Lokossou *et al*., 2009) in wild *Solanum* species. R2 and its family members are specifically LZ-NB-LRRs (with a specific type of coiled-coil domain referred to as a leucine zipper) and all are capable of recognising PiAVR2 (Lokossou *et al*., 2009), resulting in the hypersensitive response. This resistance can be overcome by *P. infestans* isolate Blue-13, which possesses the variant effector AVR2-like. AVR2-like differs from AVR2 by 13 amino acids (see **Figure 1.4**), of which 4 have been shown not to impact SdR2 recognition (Breen, 2012). Saunders *et al*. (2012) show both the virulent and avirulent forms to interact with target protein StBSL1, but only PiAVR2 mediates the further interaction of StBSL1 with SdR2. This suggests that there may be a structural difference between the PiAVR2-StBSL1 complex, and that which is formed with PiAVR2-like, which could determine whether or not R2 can bind and facilitate downstream immune signalling.

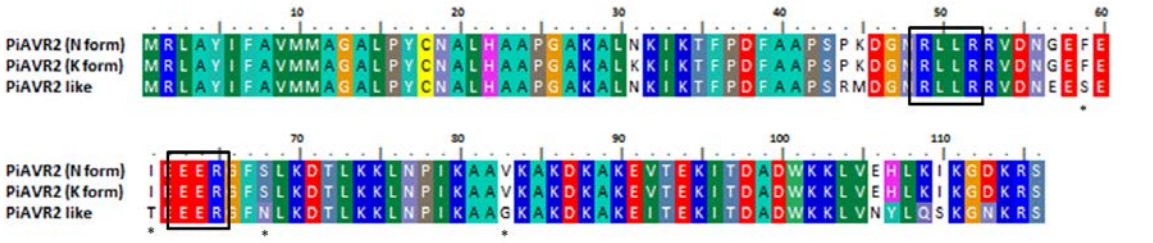

Figure 1.4 Amino acid alignment of PiAVR2 forms

Amino acid alignment of PiAvr2 showing K and N variants, both recognised by StR2, and the variant Avr2-like which evades recognition. 13 amino acid differences separate virulence from avirulance, of which 4 have been shown not to affect recognition (marked with *). The RxLR-EER motifs, required for translocation, are shown boxed.

Research on PiAVR2 interactions to date has largely focused on StBSL1. Silencing of this family member has no impact on disease progression of virulent isolates, suggestive of no direct role in promoting virulence (Breen, 2012). Mutants in brassinosteroid signalling frequently exhibit drastic phenotypes, such as curled leaves and a dwarfed stature, but NbBSL1 silencing reveals no apparent phenotype. In contrast, Breen (2012) shows that NbBSL2a and NbBSL2b silencing results in dramatic BR-deficient phenotypes, indicating that these family members have an essential function in BR signalling. This could point towards BSL1 being a decoy that has evolved purely to recognise an effector protein that would otherwise target a functional family member.

1.6 The brassinosteroid pathway

Brassinosteroids (BRs) are one of the most recently described classes of plant hormone. Structurally related to the steroids found in animal systems, they are now thought to be ubiquitous across the plant kingdom, confirmed in 61 land plant species as well as green and brown algae (Kutschera and Wang, 2012). Found at relatively low levels in the plant as a whole, their concentrations peak in reproductive tissues, and it was the growth-promoting activity of pollen extracts which led to their discovery. Grove *et al*. (1979) identified brassinolide as the active component of a growth-promoting pollen extract from *Brassica napus*, and BRs have since been implicated in cell elongation, vascular differentiation, stomatal development, senescence, biotic/abiotic stress responses and more (Clouse and Sasse, 1998). The overarching effect of BRs is positive regulation of growth and development. BR-insensitive or deficient mutants frequently exhibit extreme dwarfism (Noguchi *et al*., 1999; Nomura

et al., 2004), whilst application of BRs results in not only an increase in cell expansion but also in cell division (Nakaya *et al*., 2002).

1.6.1 Brassinosteroid signal transduction

Brassinosteroid perception at the cell surface triggers a phosphorylation cascade (shown in **Figure 1.5**), with signal transduction resulting in the accumulation of transcription factors in the nucleus, and subsequent regulation of brassinosteroid-responsive genes. A combination of approaches including forward genetics, biochemical studies and proteomics in Arabidopsis have revealed a complete pathway from BR perception to transcriptional changes (Kim and Wang, 2010), although the full extent of BR signalling is likely to be more complex, with large gene families at each step introducing the potential for redundancy/overlapping function (Kim *et al*., 2010). Additionally, family members may show tissue-specific expression levels. This has been shown for the Arabidopsis BSL family, with *BSL1* most strongly expressed in vegetative tissue, whilst *BSL2* and *3* are found to be most strongly expressed in reproductive tissue (Maselli *et al*., 2014).

Brassinosteroid binds directly to BR INSENSITIVE1 (BRI1), a transmembrane LRR-receptor like kinase (Li and Chory, 1997). This induces BRI1 dimerisation, and hetero-oligomerisation with BRI1-ASSOCIATED KINASE 1 (BAK1). The kinase domains of BRI1 and BAK1 trans-phosphorylate each other (Nam *et al*., 2002) with BAK1 thought to function in the activation of BRI1 but not in the downstream signalling (Kim and Wang, 2010). BRI1 KINASE INHIBITOR 1 (BKI1) maintains BRI1 in an inactive form until BR perception, upon which it is phosphorylated by BRI1 and dissociates (Wang and Chory,

2006). The BR SIGNALLING KINASES (BSKs), a family of cytoplasmic receptor-like kinases phosphorylated by BRI1 (Tang *et al*., 2008) positively regulate BR signalling, with a similar role played by CONSTITUTIVE DIFFERENTIAL GROWTH 1 (CDG1) (Kim *et al*., 2011). Both of these go on to interact with bri1 SUPRESSOR 1 (BSU1) and homologues BSL1, 2 and 3, with phosphorylation of these by CDG1 confirmed by Kim *et al.* (2011).

The BSU1 phosphatase de-activates the glycogen synthase kinase BRASSINOSTEROID-INSENSITIVE 2 (BIN2) (Kim *et al*., 2009), allowing the dephosphorylation of transcription factors BRASSINAZOLE RESISTANT1 (BZR1) and homologue bri1-EMS SUPRESSOR 1 (BES1) by PROTEIN PHOSPHATASE 2A (PP2A) (*Tang et al*., 2011).

These transcription factors are then free to act in gene regulation events in the nucleus. Brassinosteroid signalling results in mass transcriptional changes, such as the up-regulation of expansins and cell-wall modifying genes, regulation of other plant hormone pathways, light signalling and more (Mussig *et al*., 2002; Goda *et al*., 2002). Additionally, BZR1 represses BR biosynthetic genes such as CPD and DWF4 (Kim *et al*., 2010), contributing to brassinosteroid homeostasis by providing negative feedback. Brassinosteroid-regulated gene expression is discussed in more detail in **Chapter 4**.

1.6.2 Brassinosteroids, plant immunity and beyond

Brassinosteroids have been linked both positively and negatively with plant immunity and disease resistance. One particularly well characterised link between the two is AtBAK1. This co-receptor is shared between the brassinosteroid receptor BRI1 and several PRRs which function in the plant immune system, such as FLS2 which perceives

flagellin, and EFR which perceives the bacterial PAMP EF-Tu (Chinchilla *et al*., 2007). This is also relevant to oomycete PAMPs, with Chapparo-Garcia *et al*. (2011) identifying BAK1 homologues that are required for effective immune response to INF1.

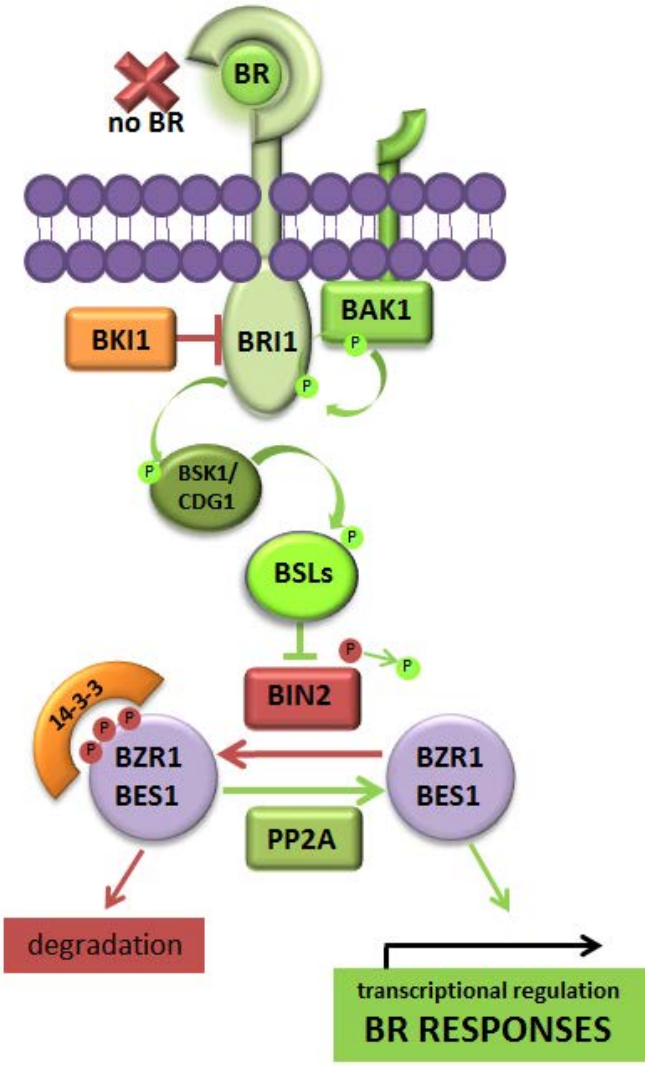

Figure 1.5 The brassinosteroid signalling pathway

Binding of brassinosteroid to the extracellular region of BRI1 triggers a phosphorylation cascade, resulting in the transcription factors BZR1 and BES1 relocating to the nucleus where they regulate expression of BR-responsive genes. Positive regulators/regulation shown in green, with negative regulators/regulation in red or orange. 'P' indicates phosphorylation.

Perception of brassinosteroid has been shown to suppress PTI; initially hypothesised to be the result of competition for BAK1. However, this has been shown not to be the limiting factor by Albrecht *et al*. (2011), with BR perception having no effect on the amount of BAK1 available for interaction with the flagellin receptor FLS2. Additionally, while BAK1 has been shown to play no role in chitin perception (Schwessinger *et al*., 2011), treatment with exogenous brassinosteroid inhibited the ROS burst associated with chitin perception by CERK1, indicating that the link between brassinosteroid and immune signalling is not solely due to this shared co-receptor (Albrecht *et al*., 2011). This effect of BR on immunity may be dose-dependent. Belkhadir *et al*. (2012) show that brassinosteroids can work both antagonistically or synergistically in flg22-induced PTI. Both the over-activity and repression of BR biosynthesis inhibited FLS2 signalling, leading the authors to stress the relevance of BR homeostasis.

De Vleesschauwer *et al*. (2012) describe the suppression of basal immunity in rice by brassinosteroid treatment. When challenged with the necrotrophic root pathogen *Pythium graminocola*, rice seedlings grown on brassinolide-containing media showed increased necrosis compared with a non-BL control. Treating plants with the BR-biosynthesis inhibitor brassinazole reduced disease severity. Further investigation revealed that not only does *P. graminocola* upregulate BR-induced genes in the plant, it also upregulates BR-biosynthesis genes, allowing the effective overthrow of the negative feedback system.

Recently, work by Lozano-Duran *et al*. (2013) and Malinovsky *et al*. (2014) has implicated the transcription factor BZR1 in BR-PTI antagonism, and describe several bHLH (basic helix-loop-helix) transcription factors upregulated by BR treatment that

act as negative regulators of PTI in Arabidopsis, such as CIB1, HBI1, and BEE2. These are discussed in more detail in **Chapter 5**.

In contrast to the antagonism between BR and PTI signalling, many examples of a positive relationship between brassinosteroids and immunity exist. Nakashita *et al*. (2003) describe the enhanced disease resistance of both tobacco and rice plants after foliar spray treatment with brassinolide. Tobacco showed increased resistance to tobacco mosaic virus (TMV), the bacteria *Pseudomonas syringae* pv. tabaci (Pst) and the fungal pathogen *Oidium* sp. Rice showed increased resistance to fungal rice blast, and bacterial blight caused by *Magnaporthe grisea* and *Xanthomonas oryzae* pv. oryzae. Ding *et al.* (2009) report that foliar spray of EBL in cucumber, *Cucumis sativum*, increased resistance to the filamentous fungi *Fusarium oxysporum*, a pathogen that infects via the roots as opposed to aerial parts. Root treatment and foliar spray both increased resistance to a comparable level, indicating that while brassinosteroids are relatively immobile whole (Symons *et al*., 2008), their presence is signalled throughout the plant.

Brassinosteroids have also been linked to the regulation of cell death. Kemmerling & Nurnberger (2008) describe the spreading regions of cell death that occur in *bak1*-deficient Arabidopsis when challenged with necrotrophic pathogens. This is also a common feature in *Nicotiana benthamiana* when *BAK1, BRI1, BSL2a* or *BSL2b* are silenced (Breen, 2012).

In addition to the relationship between brassinosteroids and biotic stress, the hormone has also been linked to abiotic stress tolerance. Dhaubhadel *et al.* (1999) show brassinosteroid treatment to increase heat tolerance in *Brassica napus* and

tomato, with increased accumulation of heat-shock proteins. Kagale *et al*. (2007) also show increased tolerance to drought and cold in Arabidopsis.

Brassinosteroids are already used in agriculture, with several commercially available growth enhancers containing 24-Epibrassinolide (EBL) as their active ingredient. Being naturally-occurring in all plants and thus already present in the food chain, they have potential as useful agrochemicals. Work by Zhua *et al*. (2009) indicates the potential of the hormone in prolonging storage life, with brassinolide treatment at a specific level reducing ethylene production, thus delaying fruit senescence. Ethylene production was reduced at lower BL concentrations, but increased at higher concentrations, hinting again at a potentially complex dose-response relationship. The use of brassinosteroid treatment post-harvest is also supported by Khripach *et al*., 2000, who show exogenous BR treatment to prolong dormancy in potato.

As an interesting aside, brassinosteroids may actually to be beneficial to human health as well as that of plants. Carange *et al*. (2011) describe the protective effects of 24-Epibrassinolide against the oxidative damage and apoptosis characteristic of neurodegenerative conditions. Malikova *et al*. (2008) also note its potential in drug development, with anti-cancer effects, and anti-viral properties have also been shown (Wachsman *et al*., 2002).

1.6.3 Brassinosteroids and hormone crosstalk

Plant hormones are organic compounds; synthesised in the plant, translocated, with the ability to cause physiological response even at very low concentrations. (Salisbury and Ross 1991). Several classes have been characterised to date including auxins,

jasmonic acid (JA), salicylic acid (SA), abscisic acid (ABA), ethylene (ET), cytokinins (CKs), gibberellic acid (GA) and the brassinosteroids (BRs) already discussed. Initially recognised for their effects on growth and development, their impact on aspects of defence and plant immunity is now coming to light, revealing a complex picture of crosstalk between signalling pathways. Although the effects of the individual hormones may overlap to a degree, their signalling pathways are not redundant, with Jallais and Chory (2010) suggesting that signals are integrated at the gene network level, rather than by crosstalk during signalling. For example, brassinosteroid-regulated genes overlap to a large extent with those regulated by auxin in their role as growth-promoting hormones (Santner and Estelle, 2009). In an analysis of BZR1 binding sites, Sun *et al*. (2010) reveal an regulatory node between brassinosteroids and the biosynthesis and signalling elements of GA, CK, ABA, ET, and JA, with a particular enrichment of auxin-associated genes.

Glazebrook (2005) contrast the two 'core' hormonal defence pathways; SA signalling (linked to defence against hemi/biotrophic pathogens) and ET/JA signalling (associated with defence against necrotrophs). These pathways are shown to be antagonistic on the whole, with defence against biotrophs compromising that against necrotrophs and vice versa. Brassinosteroids have been shown to act antagonistically with jasmonic acid, with BR treatment negating JA-induced root growth inhibition (Huang *et al.*, 2010); and also antagonising JA-promotion of zingiberene biosynthesis in tomato (Campos *et al*., 2009).

The relationship between brassinosteroid and salicylic acid is less clear-cut. Exogenous BR treatment upregulates both the SA receptor NPR1, and the transcription factor

WRKY70, both involved in positive regulation of SA signalling (Divi *et al*., 2010). However, BR treatment can antagonise SA-mediated immunity to *P. graminicola* (De Vleesschauwer *et al*., 2012). This emphasises the importance of fine-tuning within hormone cross-talk; a particular signal may have a very different outcome depending on the other stimuli present (De Bruyne *et al*., 2014).

Robert-Seilaniantz *et al*. (2011) highlight increasing evidence of plant pathogens that produce phytohormone mimics, or that intercept/hijack hormone signalling pathways and biosynthesis to facilitate the disease process. One classic example is *Agrobacterium tumefaciens*, the causal agent of crown gall disease. The pathogen inserts T-DNA into the host cell, containing genes involved in both auxin and cytokinin synthesis, to support pathogen colonisation and the formation of galls (Akiyoshi *et al*., 1983).

Hormone crosstalk is discussed in the context of brassinosteroid-regulated gene expression in **Chapter 5**.

1.7 Project Aims

The role of oomycete effector proteins remains largely unknown, with much to be discovered about their targets in the plant, and the means by which the function of these targets is manipulated to the pathogens advantage. The indirect recognition of PiAVR2 by the resistance protein R2, via the StBSL family, presents an ideal system in which to study effector biology at the molecular level. The links to the brassinosteroid pathway have the potential to reveal a novel virulence strategy of plant pathogens, which may prove relevant across a range of crop hosts.

The broad aims of this research project are:

- To further what is known about the molecular basis of PiAVR2 recognition by the resistance protein R2
- To investigate how PiAVR2 functions in pathogen virulence, by examining the role of its target protein StBSL1
- To examine similarities and differences between StBSL1 and its family members StBSL2a and StBSL2b
- To investigate the interplay between the brassinosteroid pathway and immunity in *Solanum tuberosum*

CHAPTER 2

Materials and Methods

2.1 Plant material

Nicotiana benthamiana and *Solanum tuberosum* cv Desiree plants were grown in general purpose compost, under long-day conditions consisting of a 16 hour day period at 22˚C and an 8 hour night period at 18˚C, with light intensity of 130–150 µE m−2 s−1 and humidity at 40% unless otherwise stated.

N. benthamiana was used for transient expression, silencing assays and *P. infestans* colonisation experiments at 4-5 weeks old, with *S. tuberosum* at 6-8 weeks old.

35S:AVR2 *S. tuberosum* cv. Desiree was generated by Agrobacterium-mediated transformation by FUNGEN at the James Hutton Institute. Plantlets were maintained in sterile tissue culture on 0.5 x MS (Murashige & Skoog) media plates (0.8% agar), with monthly subculturing of internodes.

2.2 Cloning and constructs

Expression constructs used in this work can be seen in **Table 2.1**. Gateway® Technology (Invitrogen) was used unless otherwise stated, utilising BP Clonase® II and LR Clonase® II, with DNA fragments cloned into the vector pDONR201 before transfer to the appropriate expression vector. Plasmid isolation was carried out using QIAprep Spin miniprep kit (Qiagen), with the resulting DNA quantified using a Nanodrop spectrophotometer (Thermo Scientific). Site-directed mutagenesis of StBSL1, 2a and 2b

was carried out using a Quik-Change II XL kit (Agilent Technologies) following the manufacturers' instructions.

ElectroMAX™ DH10b™ *E. coli* were used for vector construction and propagation, with plasmids then transferred into the *Agrobacterium tumefaciens* strain Agl1 (Lazo et al., 1991) with the additional plasmids VirG and pSOUP (van der Fits et al., 2000) for use in transient plant expression. Both *E. coli* and *A. tumefaciens* were transformed using a Micropulser™ electroporator (Bio-RAD Laboratories, Inc.) with Bio-RAD 0.1cm electroporation cuvettes. Positive clones were identified using colony PCR, using GoTaq™ Flexi DNA polymerase and GoTaq™ green master mix (both Promega). Bacteria were cultured at 37°C (*E. coli*) or 28°C (*A. tumefaciens*), supplemented with the appropriate antibiotics, either on LB agar plates or LB liquid medium with shaking. Sequencing of vector inserts was performed by the in-house sequencing facility at the James Hutton Institute. Long-term storage of *E.coli* and *A. tumefaciens* transformants was achieved by mixing 1ml of liquid culture with 1ml of 50% glycerol, flash freezing in liquid nitrogen, and storing at -80°C. Primers used in cloning, mutagenesis and sequencing can be found in **Table 2.1**.

2.3 Agrobacterium-mediated transient expression

Glycerol stocks were streaked out onto YEB plates containing the appropriate antibiotics, and incubated at 28°C for 48 hours. Bacterial colonies were used to inoculate liquid YEB media and incubated overnight at 28°C with shaking. Cultures were spun down at 1000 x g for 10 minutes, and the bacterial pellet resuspended in

10mM MES 10mM $Mgcl_2$ buffer. OD_{600} was adjusted to 0.5 for cell death assays, or to 0.1 for *P. infestans* colonisation assays, with acetosyringone added at 200µM. Leaves of *N. benthamiana* or *S.tuberosum* cv. Desiree were pressure infiltrated on the abaxial surface, using a 1ml syringe after wounding with a needle. *A. tumefaciens* constructs used for transient expression in this study are detailed in **Table 2.2**.

2.4 *P. infestans* colonisation assays

P. infestans strain 88069 constitutively expressing td Tomato (tdT) was grown on rye agar supplemented with 20 µg/ml geneticin (Thermo Fisher Scientific Ltd.) as a selective antibiotic. To harvest sporangia, plates were flooded with 5ml sterile distilled water before scraping with a spreader into a 50ml falcon tube. This suspension was spun down at 1000 x g for 10 minutes, supernatant discarded, and the pellet resuspended in fresh distilled water. Sporangia were quantified using a haemocytometer, and adjusted to a concentration of 50,000/ml. 10µl droplets were pipetted onto the abaxial surface of detached leaves, maintained in sealed boxes with moist tissue. Boxes were kept in darkness for the first 24 hours to reduce UV degradation of sporangia. Lesions were measured at the widest point 7 days post infiltration. When used in combination with Agrobacterium-mediated transient expression, *P. infestans* was inoculated 24 hours post-infiltration of the *A. tumefaciens* suspension. Instead of inoculating detached leaves, plants were kept intact in propagators, with sporangia suspension pipetted onto the adaxial side of the leaf slightly away from the original wound site used for infiltration.

Table 2.1 Primers used in this study for sequencing, cloning and mutagenesis
For primer use, 'S' refers to sequencing, 'C' to cloning, and 'M' to mutagenesis.

Primer name	Primer sequence (5' - 3')	Use	Source
pDonr201_F	TCGCGTTAACGCTAGCATGGATCTC	S	Hazel McLellan
pDonr201_R	GTAACATCAGAGATTTTGAGACAC	S	Hazel McLellan
EES-F	TGCTGCCCGACAACCACTACC	S	Hazel McLellan
RTL2-P	AAGGAAGTTCATTTCATTTGGAGAGGA	S	Hazel McLellan
RTL2-M	CAACACATGAGCGAAACCCTATAAGAA	S	Hazel McLellan
attB_F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACC	S	Hazel McLellan
attB_R	GGGGACCACTTTGTACAAGAAAGCTGGG	S	Hazel McLellan
StBSL1_CtermF	AAAGCAGGCTTCATGGTGAGGCAATTGTCA	C	this study
StBSL1 FL _R	GAAAGCTGGGTATTAAATATAGGCAAGTGAGCT	C	Breen (2012)
StBSL1 FL _F2	ATCTGGTAACTGTCAGTGGCA	S	Breen (2012)
StBSL1 FL _R2	TGCTCAACTGAATTTATTGAC	S	Breen (2012)
StBSL1 FL _F3	TCTGGTTGCAGAAAATTCTCC	S	Breen (2012)
StBSL1 H648V F	CATTTGATAAGAGGGAATGTTGAAGCTGCTGATATTAATGC	M	this study
StBSL1 H648V R	GCATTAATATCAGCAGCTTCAACATTCCCTCTTATCAAATG	M	this study
StBSL1 S836D F	CCTTTGCCGCCTCCTCTTCTAGACCCTGAAACGTCTC	M	this study
StBSL1 S836D R	GAGACGTTTCAGGGTCTAGAAGAGGAGGCGGCAAAGG	M	this study
StBSL1 S836A F	TTTGCCGCCTCCTCTTCTAGCCCCTGAAACG	M	this study
StBSL1 S836A R	CGTTTCAGGGGCTAGAAGAGGAGGCGGCAAA	M	this study
StBSL2_CtermF	AAAGCAGGCTTCATGGTGCGGCAGCTTTCG	C	this study
StBSL2_FL_R	GAAAGCTGGGTCTTAAGTCCAAGCAACAGAAC	C	Breen (2012)
StBSL2_F1	CTAGGATGACCCCAATAGGA	S	Breen (2012)
StBSL2_F2	CTGTTTTTGTTAATGCTCGGC	S	Breen (2012)
StBSL2_R1	CCATAGTAATAGGGCGTTGGA	S	Breen (2012)
StBSL2_R2	TTAGGAACAGTGTTGATGGACA	S	Breen (2012)
StBSL2 H769V_F	CATCTAATTCGTGGGAACGTTGAAGCTGCTGATATTAATGC	M	this study
StBSL2 H769V_R	GCATTAATATCAGCAGCTTCAACGTTCCCACGAATTAGATG	M	this study
StBSL3_CtermF	AAAGCAGGCTTCATGGTACGACAGCTTTCT	C	this study
StBSL3_FL_R	AGAAAGCTGGGTCTAAGTCCAAGCAAAAGAACCTCGATCG	C	Breen (2012)
StBSL3_5' Ra	TTCCATAACTGACTCGCCGGCCTTC	S	Breen (2012)
StBSL3_5' Ra_R2	CCAGTACAACAGAACCA	S	Breen (2012)
StBSL3_origsec_F	GGGATGGTGGAGCAGAGAC	S	Breen (2012)
StBSL3 H767V_F	CATTTAATACGTGGGAACGTTGAAGCTTCAGATATAAATGC	M	this study
StBSL3 H767V_R	GCATTTATATCTGAAGCTTCAACGTTCCCACGTATTAAATG	M	this study

Table 2.2 Constructs used for Agrobacterium-mediated transient expression

Agrobacterium strain	Expression vector	Gene insert	Source
Agl1 VirG pSOUP	pB7WGF2	StBSL1	Breen (2012)
Agl1 VirG pSOUP	pB7WGF2	StBSL1 Ct	this study
Agl1 VirG pSOUP	pB7WGF2	StBSL1 H648V	this study
Agl1 VirG pSOUP	pB7WGF2	StBSL1 H648V Ct	this study
Agl1 VirG pSOUP	pB7WGF2	StBSL1 S836A	this study
Agl1 VirG pSOUP	pB7WGF2	StBSL1 S836D	this study
Agl1 VirG pSOUP	pB7WGF2	StBSL2a	Breen (2012)
Agl1 VirG pSOUP	pB7WGF2	StBSL2a Ct	this study
Agl1 VirG pSOUP	pB7WGF2	StBSL2a H767V	this study
Agl1 VirG pSOUP	pB7WGF2	StBSL2a H767V Ct	this study
Agl1 VirG pSOUP	pB7WGF2	StBSL2b	Breen (2012)
Agl1 VirG pSOUP	pB7WGF2	StBSL2b Ct	this study
Agl1 VirG pSOUP	pB7WGF2	StBSL2b H769V	this study
Agl1 VirG pSOUP	pB7WGF2	StBSL2b H769V Ct	this study
Agl1 VirG pSOUP	pB7WGF2	StHBI1-like	this study
Agl1 VirG pSOUP	pB7WGF2	PiAVR2	Gilroy et al. (2011)
Agl1 VirG pSOUP	pGRAB	PiAVR2	Gilroy et al. (2011)
Agl1 VirG pSOUP	pGRAB	PiAVR2-like	Gilroy et al. (2011)
Agl1 VirG pSOUP	pKGW	R2-like	Gilroy et al. (2011)
Agl1 VirG pSOUP	pKGW	Sto1	Vleeshauwers *et al*. (2008)
Agl1 VirG pSOUP	pGRAB	Ipi01	Vleeshauwers *et al*. (2008)
Agl1 VirG pSOUP	pK7WG2	R3a	Bos *et al*. (2010)
Agl1 VirG pSOUP	pGRAB	$AVR3a^{KI}$	Bos *et al*. (2010)
Agl1 VirG pSOUP	pCB302-3	INF1	Eleanor Gilroy
Agl1 VirG pSOUP	pJL3	P19	Eleanor Gilroy
LBA4404	pBIN TRV2b	GFP	Gilroy *et al*. (2007)
LBA4404	pBIN TRV2b	BAK1	Heese *et al.* (2007)
LBA4404	pBIN TRV2b	BRI1	Ana Confraia
LBA4404	pBIN TRV2b	BSL1 5'	Breen (2012)
LBA4404	pBIN TRV2b	BSL1 3'	Breen (2012)
LBA4404	pBIN TRV2b	BSL2a	Breen (2012)
LBA4404	pBIN TRV2b	BSL2b	Breen (2012)
LBA4404	pBIN TRV	RNA1	Liu *et al*. (2002)

2.5 Virus-induced gene silencing (VIGS)

Virus-induced gene silencing (VIGS) constructs were obtained from Dr. Susan Breen & Dr. Eleanor Gilroy. These constructs consisted of short PCR fragments of the gene targeted for silencing, cloned in antisense into pBinary Tobacco Rattle Virus (TRV) vectors (Liu *et al.*, 2002). A TRV construct expressing GFP was used as a control (Gilroy *et al*., 2007). The BAK1 construct was obtained from John Rathjen (Heese *et al*. 2007). To achieve transient silencing, *N. benthamiana* plants at the four- leaf stage were pressure infiltrated with a mixture of *A. tumefaciens* strain LBA4404 containing TRV RNA1 at a final OD_{600} of 0.25, and pYL156 containing antisense fragments corresponding to each gene of interest, at a final OD_{600} of 0.5. The two largest leaves were fully infiltrated, and viral infection allowed to progress systemically through the plant for 2-3 weeks before use in downstream assays. *A. tumefaciens* constructs used for VIGS are detailed in **Table 2.2**.

2.6 Yeast-2-Hybrid analysis

Yeast-2-Hybrid (Y2H) screening was carried out using the ProQuest™ system (Invitrogen). Genes of interest were cloned into pDEST32 (Bait) and pDEST22 (Prey) vectors and used in pairs to transform the yeast strain MaV203 according to the Small Scale Yeast Transformation protocol from the Invitrogen ProQuest handbook. Positive transformants were identified by selection on plates lacking Leucine and Tryptophan. These were then screened for HIS3 induction and URA3 induction by plating on the appropriate drop-out media, and for β-galactosidase induction by X-gal assay according to the manufacturers' instructions. In this system, a strong interaction between bait and prey results in induction of all three reporter genes. Moderate

interactions induce HIS3 and β-galactosidase, whilst weak interactions typically induce the HIS3 reporter gene only. Bait constructs pDEST32 PiAVR2 and StBSL1, prey construct pDEST22 StBSL1 and empty vector controls were provided by Susan Breen (Breen, 2012). The prey construct pDEST22 StBSL1 H648V was generated in this study using gateway technology (see **Section 2.2**).

2.7 Hormone and PTI elicitor treatments

Epibrassinolide (EBL) (Sigma-Aldrich Co.) was first solubilised at 20mM in ethanol. EBL treatment was carried out by foliar spray at 50µM in distilled water, with the addition of 0.5% Tween-20 (Sigma-Aldrich Co.). Distilled water with ethanol and Tween-20 was used as a negative control.

Culture filtrate was obtained from Dr. Steve Whisson, The James Hutton Institute, UK. Briefly, this was prepared by inoculation of sterile amended lima bean broth with *P. infestans* strain 88069. The media was left to incubate in darkness at room temperature for 5 days, then filtered through 70 µm nylon mesh (BioDesign CellMicroSieves; Fisher Scientific) to remove mycelium. Culture filtrate was then filter sterilized through a 0.20 µm syringe filter (Millipore, UK). This was used to pressure infiltrate leaves of *S. tuberosum* cv. Desiree, or *N. benthamiana* by wounding lightly with a needle before infiltrating with a 1ml syringe. Uninoculated media was used as a control. Culture filtrate was used as an oomycete 'PAMP cocktail' – containing an assortment of *P. infestans* secretions that result in a plant immune response. This was used in PTI timecourses, where leaf tissue was harvested over a period of 24 hours.

Flg22 peptide (Peptide Protein Research Ltd.) was dissolved at 40µM in sterile distilled water before infiltration of leaves in the same manner, with pure sterile distilled water used as a control. Again this treatment was used for induction of a PTI response, with leaf tissue collected over a timecourse of 24 hours.

2.8 Western blotting

Leaf tissue samples were taken 48 hours post-infiltration with *A. tumefaciens* suspensions, by excising 3 x 1cm leaf discs and immediately freezing in liquid nitrogen. Protein extraction was carried out by boiling ground leaf tissue samples in 300µl of 2xSDS sample buffer (100mM Tris-Cl pH6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol) with freshly added 200mM DTT (Sigma-Aldrich Co.) at 95°C for 10 minutes, followed by centrifugation at 13,000 x g for 5 minutes. Samples were separated on 4-12% Bis-Tris PAGE gels with MES buffer using an X-Blot Mini Cell (all Thermo Fisher Scientific Inc.), followed by transfer to nitrocellulose membrane (Amersham Protran premium 0.45µm NC, GE Healthcare Life Sciences) using an X10 Blot Module (Thermo Fisher Scientific inc.) following manufacturers' instructions. Membranes were then stained using ponceau solution to visualise relative protein loading. Membranes were blocked in 4% milk in 1 x phosphate-buffered saline with 0.1% Tween (1xPBS-T) by shaking overnight at 4°C. Membranes were then incubated for 2 hours with a polyclonal GFP antibody raised in rabbit (Santa Cruz) at 1:1000 in 4% milk 1xPBST, before washing 3 x 5 minutes in 1xPBST. A secondary incubation with anti-rabbit IgG HRP (Sigma-Aldrich Co.) at 1:5000 was carried out for 50 minutes, before a further 6 x 5 minute washes. Signal was detected using Amersham ECL Prime as described in the manufacturers' instructions, on Amersham Hyperfilm ECL film (both GE Healthcare Life

Sciences). Films were developed with a Compact X4 Automatic Processor (Xograph Healthcare Ltd.)

2.9 Quantification of stomata

Epidermal leaf prints were obtained by pressing leaf sections onto microscope slides with opaque adhesive tape. 10µl of acetone was applied to the tape, and allowed to dry before pressing leaf sections onto the slides. A Leica DMLF5 compound microscope was used to view the epidermal leaf prints, with number of stomata and number of epidermal cells counted per 0.5mm^2. Multiple prints were scored, representing at least 3 leaves per plant across 3 plants or more. For confocal microscopy, leaf tissue was cut into small sections approximately 1cm^2, and stained with Calcofluor White (Sigma-Aldrich Co.) for 10 minutes before mounting sections in water on a microscope slide. Images were acquired on a Zeiss 710 confocal microscope with a Zeiss Epiplan APO X20/0.6 lens using 405nm excitation and collecting emissions between 417 and 480 nm.

2.10 Microarray analysis

Solanum tuberosum cv. Desiree plants were grown in controlled conditions within a growth cabinet (Snijders Scientific) at 20°C, with 16 hours light, 8 hours dark at 70% humidity. 8-week old plants were treated with a foliar spray of 50µM Epibrassinolide (Sigma) or a mock control containing distilled water and ethanol only. Epibrassinolide was first solubilised at 20mM in ethanol before dilution in distilled water. Leaf tissue was collected at 3 and 24 hours, and immediately frozen in liquid nitrogen and stored

at -80°C until sample processing. RNA extraction was carried out as described above, with samples assessed for purity using a Nanodrop (Thermo Scientific). Integrity of RNA samples was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.). RNA was labelled using the Agilent Two Colour Low Input Quick Amp labelling kit (following manufacturer's instructions), and cRNA hybridised to custom JHI *Solanum tuberosum* 60K microarrays overnight. Arrays were washed and scanned using an Agilent G2505B scanner, followed by data extraction using Agilent FE software and analysis in Genespring (Agilent Genomics). Statistically significant changes in gene expression between treatments was identified using volcano filtering (T-test p-value <0.05; fold change >2x). Quantitative RT-PCR was used to validate transcripts of interest from these microarrays.

2.11 MapMan analysis of microarray data

Transcript identifiers (DMP number) and the associated log2 fold-change ratios were imported into Mapman software version 3.5.1R2 (Thimm *et al*., 2004) Data was allocated to BINS using the *Solanum tuberosum* mapping Stub_PGSC_DM_v3.4 (downloaded from mapman@mpimp-golm.mpg.de). BINS that behave with significant difference to the average response were identified by Wilcoxon Rank Sum Test, with p values corrected by Benjamini Hochberg correction. Over-representation analysis was carried out using Fishers exact test with Benjamini Hochberg correction, using statistical tools built into the Mapman platform.

2.12 Gene expression analysis (qRT-PCR)

RNA was isolated from plant tissue with the RNeasy Plant Mini Kit (Qiagen) according to the manufacturers' instructions, including the on-column DNase treatment. RNA was quantified using a Nanodrop 1000 (Thermo Scientific) and cDNA synthesised using Superscript II (Qiagen) with oligo dT primers (Eurofins MWG Operon). qRT-PCR was performed using PrecisionPLUS SYBR green Mastermix (Primer Design Ltd.) and Maxima SYBR green qPCR Mastermix (Thermo Scientific). Detection and data acquisition was achieved with a Chromo4^{TM} real-time detector with MJ Research PTC-200 thermal cycler and Opticon Monitor 3.1.32 software (all Bio-Rad Laboratories Inc.). Reactions were incubated at 95° for 15 minutes, before 40 cycles of 95°C for 15 seconds, 60°C for 1 minute, and plate reading. A melting curve was added between 58°C and 95°C, with plate read every 1°C and hold for 5 seconds. Data analysis was performed using the comparative C_T method as described in the ABI PRISM 7700 Sequence Detection System User Bulletin #2 (Applied Biosystems). Expression was normalised to a housekeeping gene (Ubiquitin for *S. tuberosum*, or Elongation Factor 1α for *N. benthamiana*). All primers used for qRT-PCR are shown in **Table 2.3**. Primer design was based on Applied Biosystems criteria for qRT-PCR primers described in the handbook, in addition to sequence information from Sol Genomics Network (Fernandez-Pozo *et al*., 2014) at www.solgenomics.net. Primer design was also facilitated by the use of Primer3 (Untergasser *et al*. 2003; Koressaar and Remm, 2007) at http://primer3.ut.ee/ and NetPrimer sotware (PREMIER Biosoft) at http://www.premierbiosoft.com/netprimer/.

2.13 Statistical analysis and desktop publishing

All statistical analysis was carried out in SigmaPlot (Systat Software Inc.) unless otherwise stated. Cell death and pathogen growth assays were assessed for statistical significance using one way analysis of variance (ANOVA). This was combined with the post hoc Holm-Sidak test to examine the statistical signifiance of differences in means, in a pairwise manner, within the larger dataset. Error bars displayed for pathogen growth/cell death assays represent standard error of the mean (SEM), to reflect the accuracy of the population mean.

Figures were generated using Microsoft Excel, Adobe Illustrator and Adobe Photoshop.

Table 2.3 Primers used for qRT-PCR in this study

Published references or sources are noted where appropriate. For primers designed as part of this work, a transcript number is provided.

Primer name	Primer sequence	Reference/Source
NbEF1a_F	TGGACACAGGGACTTCATCA	Eleanor Gilroy
NbEF1a_R	CAAGGGTGAAAGCAAGCAAT	Eleanor Gilroy
qRT_NbAcre31_F	AATTCGGCCATCGTGATCTTGGTC	Nguyen *et al.* (2010)
qRT_NbAcre31_R	GAGAAACTGGGATTGCCTGAAGGA	Nguyen *et al.* (2010)
qRT_NbWRKY7_F	CACAAGGGTACAAACAACACAG	Ishihama *et al.* (2011)
qRT_NbWRKY7_R	GGTTGCATTTGGTTCATGTAAG	Ishihama *et al.* (2011)
St-TUB_F	CAAATGTGGGATGCCAAGAA	Eleanor Gilroy
St-TUB_R	AGCTGTCAGGTAACGTCGTCCATGA	Eleanor Gilroy
StUBIfwd	ACACCATTGATAATGTCAAGGCTAAG	Eleanor Gilroy
StUBIrev	GCCATCCTCCAATTGCTTTC	Eleanor Gilroy
PiAvr2old F	CCCAAGCCCTAAAGACGGC	Breen (2012)
PiAvr2old R	TCACCCTTAATTTTGTAATGC	Breen (2012)
StSlNt_Cyp71_For	TGTCATCAACGAGCACAAGA	Heese *et al*. (2007)
StSlNt_Cyp71_Rev	TGGTGATGGGAAATTGAAGA	Heese *et al*. (2007)
qRT_StWRKY7F	CCAACTGGAAGCAACAACAA	Hazel MacLellan
qRT_StWRKY7R	CCTGATTAGAATGATTAGCCAACA	Hazel MacLellan
qRT_StACRE31-F	CAGGATGAATCGGATCTGAAA	Hazel MacLellan
qRT_StACRE31-R	CGGCAATCCCAATTTCTCTA	Hazel MacLellan
StFLS2_qF	GCTAGGTTACGCTTGGGAAAG	PGSC0003DMT400021384
StFLS2_qR	CGTCCAGTCATCCTCCACTT	PGSC0003DMT400021384
StEXP8_F	TGTTGGAGGTGCTGGTGATA	PGSC0003DMT400042943
StEXP8_R	AATTTTGGCCCCAATTTCTT	PGSC0003DMT400042943
StDWF5_F	ATATCCTCGCATTGGCAAAC	PGSC0003DMT400015184
StDWF5_R	ATAGGGAGGAGTCCCCAAGA	PGSC0003DMT400015184
StSTDH_F	TGCAACATGCCCACATTATC	PGSC0003DMT400002567
StSTDH_R	TGCTCCTTCCCATCAAGTAAA	PGSC0003DMT400002567
StP69F_F	TCACCTTCCACGGAACTGTA	PGSC0003DMT400009990
StP69F_R	CTCGTGAGGAAAATGAAGCA	PGSC0003DMT400009990
StBHLH7#2_F	TGAGCTTGTGACTCGGGCAAT	PGSC0003DMT400001888
StBHLH7#2_R	CCTCCGTGTCCAACTCCAACT	PGSC0003DMT400001888
StChlor43054_F	GCCGATCCAGAAACTTTTGC	PGSC0003DMT400043054
StChlor43054_R	CAGCTTCACCGAACTTGACA	PGSC0003DMT400043054
StGibOX_qF	AGTCCCACCTGATCCCTACT	PGSC0003DMT400054348
StGibOX_qR	CCATTGACACCCTGGCTTTC	PGSC0003DMT400054348
StSAUR4187_qF	CAATGCCTCCTTCGACAATCC	PGSC0003DMT400004187
StSAUR4187_qR	ACACAACTTCTTCGCATGGAA	PGSC0003DMT400004187

CHAPTER 3

StBSL1 as a susceptibility factor in *Phytophthora infestans* infection

3.1 Introduction and Experimental Aims

Previous work by Breen (2012) and Saunders *et al.,* (2012) describes the indirect recognition of PiAVR2 by R2, with StBSL1 as the intermediate target. StBSL1 is shown to be essential for PiAVR2 recognition, with transient silencing in *N. benthamiana* leading to a reduction in the HR. Transient silencing appears to have no other effect on the plant; there is no phenotype apparent, and this leads the authors to suggest the possibility of BSL1 acting as a 'decoy', with no direct role of StBSL1 in the plants biology other than in the detection of PiAVR2. Its function would therefore be to reduce the interaction between the effector and its true target, and facilitate recognition and immune signalling. Additionally, PiAVR2 has also been shown to interact with StBSL1 protein family members StBSL2a and StBSL2b. These proteins appear to be more crucial to the plants physiology, with transient silencing causing a severe developmental phenotype. This family of protein phosphatases with kelch-like domain (PPKL) proteins is implicated in brassinosteroid pathway signalling, with increasing evidence that this hormone signal transduction pathway has an antagonistic role in plant immunity.

Based on this knowledge, the work detailed in this chapter aims to:

- Further what is known about the molecular basis of PiAVR2 recognition by R2
- Investigate the effect that PiAVR2 has on target protein StBSL1
- Determine the function of effector and target in *P. infestans* virulence
- Examine similarities and differences between StBSL1 and its family members StBSL2a and StBSL2b.

3.2 Role of BSL1 in AVR2 recognition by R2

StBSL1 is a putative phosphatase, and its requirement in the AVR2-R2 hypersensitive response raises the question: is phosphatase activity required for the recognition of AVR2? Data from Eleanor Gilroy (see **Figure 3.1**) indicates that this may be the case. Okadeic acid is a potent inhibitor of phosphatase activity (PP1 and PP2C subgroups in particular), and is shown here to suppress the AVR2-R2 HR, while having minimal impact on a control HR elicited by *P. infestans* RXLR effector IpiO and *S. stoloniferum* resistance protein Rpi-Sto1. The observation that a control HR is unaffected means that phosphatase activity is not required for the hypersensitive response *per se*, and suggests that specifically an element of AVR2 recognition requires this enzymatic activity. This may be the activity of BSL1 itself, or potentially the activity of another phosphatase either involved in the interaction between AVR2, BSL1 and R2, or in the signal transduction from R2 to immune activation.

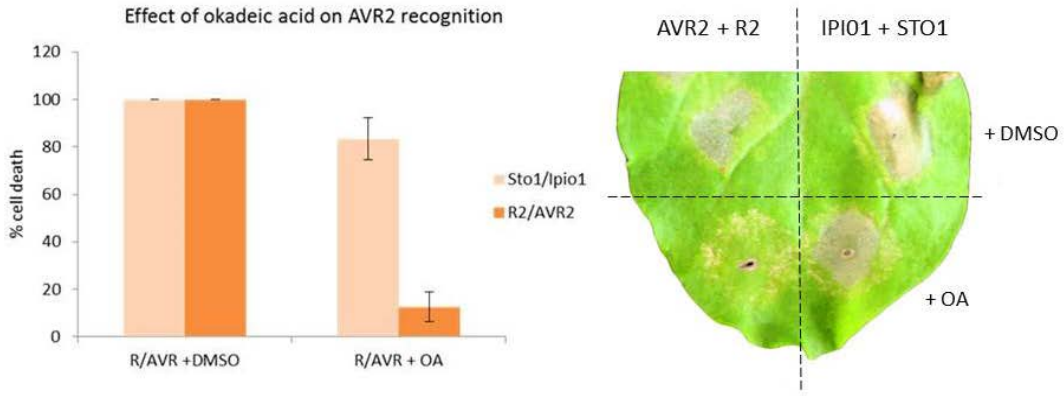

Figure 3.1 Effect of okadeic acid on AVR2 recognition by R2
Data courtesy of Eleanor Gilroy. Error bars indicate SEM. HR lesions were scored at 5dpi.

To investigate this further, site-directed mutagenesis was used to generate BSL1 variants with altered phosphatase activity. These were designed to disrupt the active site to impede substrate binding, and additionally to disrupt protein activation by modifying the phosphorylation site. The Pfam protein family database (Finn *et al*., 2014) was used to identify an active site within the phosphatase domain, predicted to be a histidine residue at position 648. This was mutated to a valine residue to generate a putative 'phosphatase-dead' mutant StBSL1 H648V. Predicted domains of StBSL1 are shown in **Figure 3.2.** This histidine residue is also conserved in mammalian PP1, with a mutagenesis study by Zhang *et al*. (1996) indicating that this residue is required for the catalytic activity in its role as a proton donor. This mutated StBSL1 was later confirmed as being phosphatase-dead in a biochemical assay by colleague Shaista Naqvi.

BSL1 wild-type and H648V were transiently expressed with AVR2 and R2 to observe any changes in the hypersensitive response (see **Figure 3.3**). To determine whether any effects were specific to AVR2 recognition, recognition of IpiO by Rpi-Sto1 was used

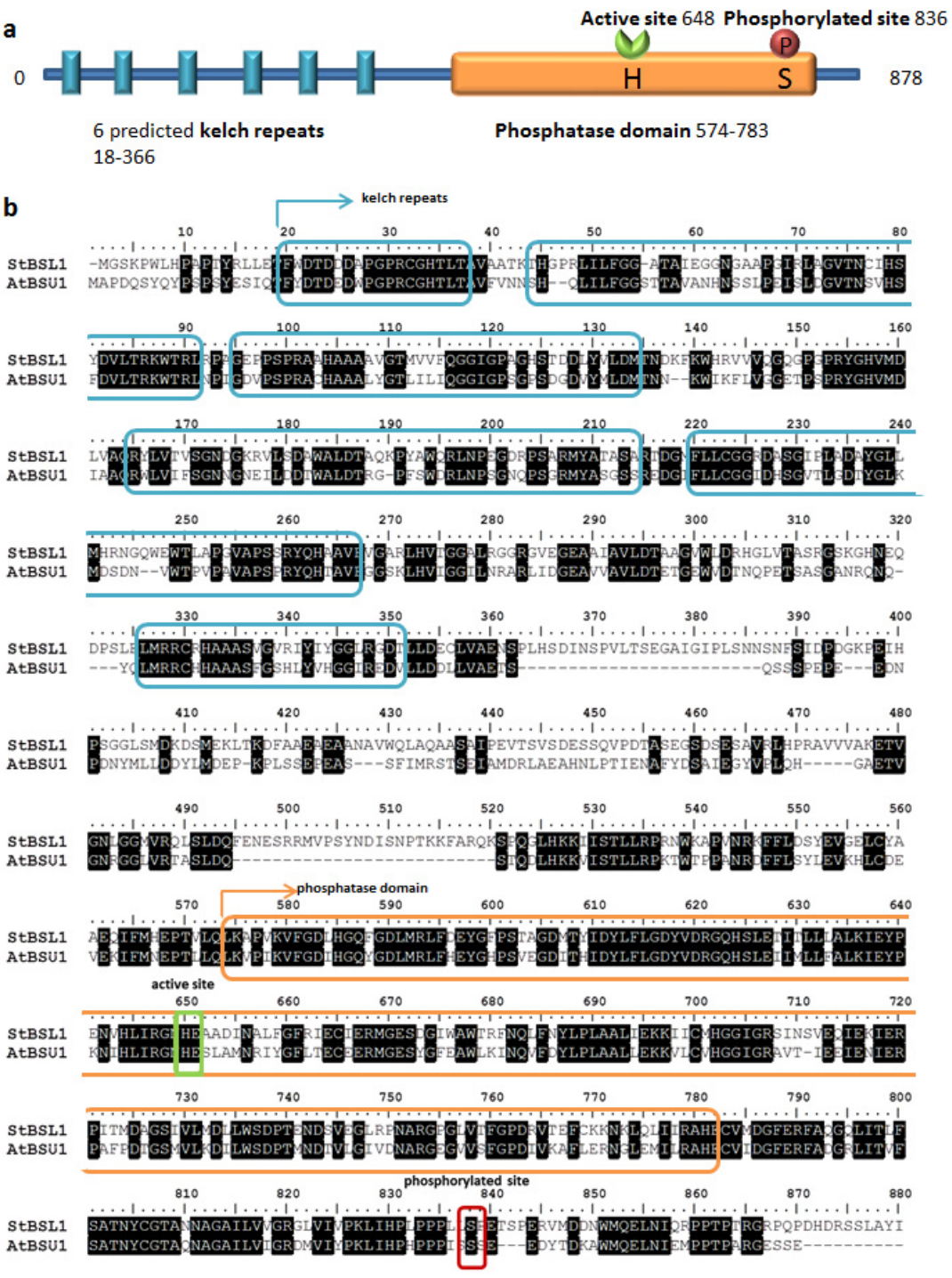

Figure 3.2 Predicted domains, phosphorylation site and active site of StBSL1
a. Domains and active site prediction by Pfam. Phosphorylated site predicted on basis of similarity to AtBSU1 site phosphorylated by AtCDG1 (Kim *et al.*, 2011) **b.** Amino acid alignment of StBSL1 with AtBSU1. Green box indicates predicted active site (Pfam) and red box indicates AtBSU1 residue phosphorylated by AtCDG1. Location of kelch repeats and phosphatase domain predicted in StBSL1 by Pfam. Amino acid alignment constructed using ClustalW in BioEdit.

as a control. The first point of interest is the significant boost to the HR seen when wild-type StBSL1 is co-expressed with the effector and resistance gene, with almost ninety per cent of sites showing cell death compared to around fifty per cent with the empty vector control. StBSL1 has been previously shown to be required for the AVR2-R2 HR by Saunders *et al*. (2012) and Breen (2012), with transient silencing of BSL1 in *N. benthamiana* abolishing cell death. The increased HR seen with wild-type StBSL1 supports this and suggests that StBSL1 is rate-limiting in the recognition of AVR2 by R2. To visualise this increase, it was necessary to adjust the *A. tumefaciens* OD_{600} for AVR2 and R2L constructs down to 0.1 instead of 0.5 to reduce the strength of the HR, and to score the number of sites showing cell death at 3 days post-infiltration rather than the usual later time-point of 5 or 7 dpi. This explains the relatively low percentage cell death in the control; AVR2 + R2 would commonly provide approximately seventy to eighty per cent cell death, but the level is reduced to fifty per cent in this experiment.

Figure 3.3 also shows the mutant StBSL1 H648V to have a significant suppressive effect on the AVR2-R2 hypersensitive response, with only twenty per cent of sites showing a positive HR, compared to almost fifty percent with the empty vector. This suppression was shown to be specific for the AVR2-R2 HR, with StBSL1 H648V having no effect on the control HR elicited by Sto1 and Ipi01. This implies that phosphatase activity of StBSL1 is required for the recognition of AVR2. It may be that desphosphorylation of R2 is required for immune signalling. Additionally, the observation that StBSL1 H648V actually suppresses the HR, as opposed to having no effect, suggests that this phosphatase-dead variant may be titrating the effector away from endogenous

NbBSL1, resulting in a dominant negative effect thus less recognition by R2. This would require AVR2 to maintain its interaction with BSL1 even when this site is mutated.

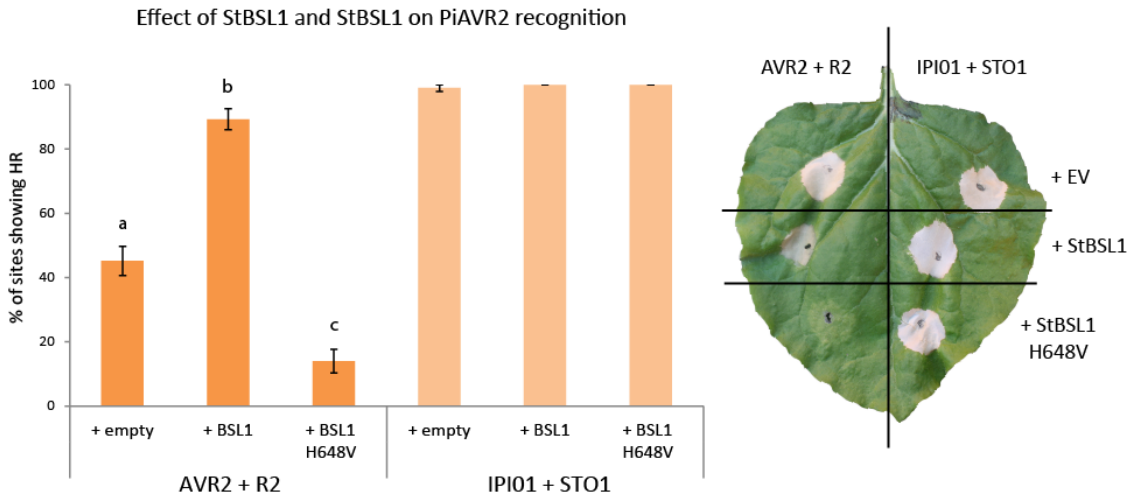

Figure 3.3 AVR2-R2 hypersensitive response with StBSL1 variants
Effector, resistance gene and either empty vector control or BSL1 variant were transiently co-expressed in *Nicotiana benthamiana,* with HR lesions scored after 3 days. Error bars indicate SEM. a≠b p≤0.001, a≠c p≤0.001 in one-way ANOVA (Holm-Sidak). Data combines at least 3 biological replicates.

Alternatively, BSL1 may be present in the plant cell as a dimer. When BSL1 H648V is present, it may interact with the wild-type BSL1 and result in a non-functional dimer. This would allow the suppression of the HR without any direct interaction between AVR2 and StBSL1 H648V. A preliminary experiment using the yeast-2-hybrid system (**Figure 3.4**) supported the possibility of dimerisation, showing interaction between both StBSL1 and itself, and between StBSL1 and BSL1 H648V.

The yeast-2-hybrid data also suggests a lack of interaction between PiAVR2 and the mutant BSL1 H648V. However, a repeat of this suggested the opposite result, with positive colonies growing on the HIS3 plate, so the result is inconclusive. This

preliminary data requires repeating in full, with expansion to include each pairing in both orientations of bait and prey, as well as confirming stable expression of constructs in the yeast system to validate any negative results.

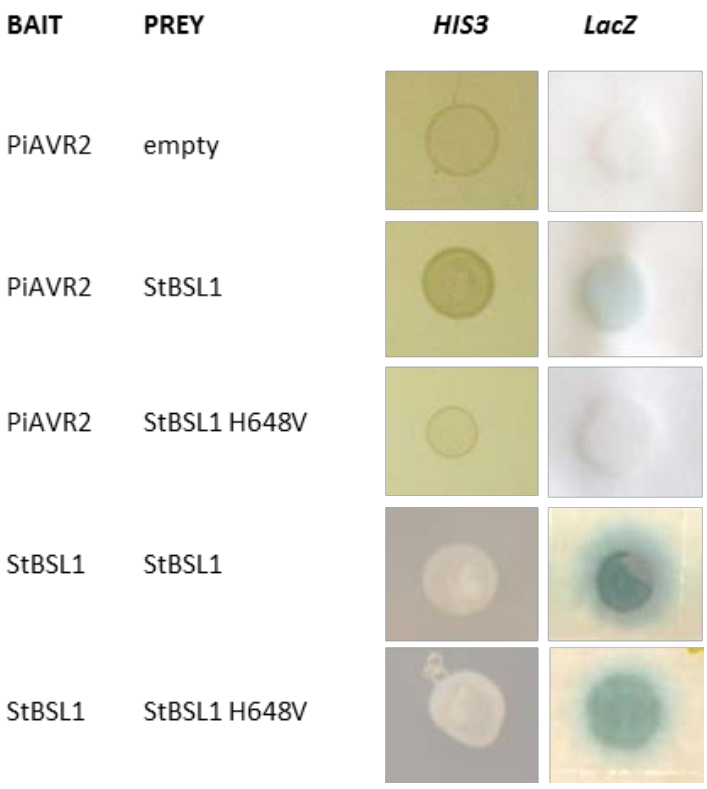

Figure 3.4 Yeast-2-Hybrid analysis with BSL1 wild-type and phosphatase-dead
Left hand column represents HIS3 reporter gene activation status (growth on reporter plate indicates positive interaction), and right-hand column represents LacZ reporter gene activation (blue colouration indicates positive interaction). Figure displays representative colonies from the six plated for each combination.

To investigate StBSL1 activity further, an attempt was made to identify the potential phosphorylation site required for protein activation. The sequence was aligned with that of AtBSU1, the best characterised member of the BSL family in Arabidopsis. This

has been shown to be activated by phosphorylation at S736 by the kinases AtCDG1 and CDL1, resulting in increased binding to its substrate AtBIN2 (Kim *et al*., 2011). This serine is part of a conserved region of the phosphatase domain in both proteins (see **Figure 3.2**) and corresponds to S836 in StBSL1. This was mutated to an alanine, to prevent phosphorylation at this site and achieve constitutive inactivation (StBSL1 S836A). An additional variant was created by mutating this serine to an aspartate, to create a phospho-mimetic protein which should behave as if constitutively active (StBSL1 S836D). This was transiently expressed with AVR2 and R2 to determine any effects on effector recognition, shown in **Figure 3.5**.

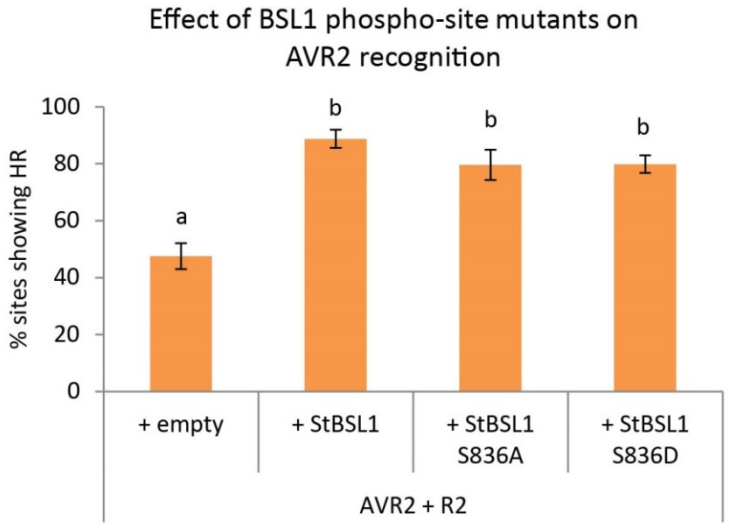

Figure 3.5 Effect of BSL1 phospho-site mutants on AVR2 recognition
AVR2, R2 and either an empty vector control or BSL1 variants were expressed transiently in *Nicotiana benthamiana*, with HR lesions scored after 3 days. a≠b p≤0.001 in one-way ANOVA (Holm-Sidak method). Data combines at least 3 biological replicates.

Unfortunately, no alteration in the hypersensitive response was seen with the phosphorylation site mutants StBSL1 S836A or S836D, with the HR boosted to the same level as with wild-type StBSL1. This suggests that another serine residue may be

the key phosphorylation site required for StBSL1 activation. Or, perhaps this phosphorylation site is important for brassinosteroid signalling, but entirely separate to R2 recognition of AVR2. Kim *et al*. (2011) identify two other phosphorylated sites on AtBSU1; S395 and S444. While mutation of these sites had no bearing on AtBSU1 phosphorylation by AtCDG1, they could potentially be phosphorylated by an alternative kinase and lead to differential activity of the protein. S444 corresponds to an AtBSL1 phospho-site identified at S491 by Sugiyama *et al*. (2008). BSL1 is highly conserved between Arabidopsis and potato, and the corresponding serine in the potato sequence, S489, may be the next logical site to investigate in StBSL1 activity. The web-based tool NetPhosK 1.0 (Blom *et al*. 2004) can be used to predict kinase-specific phosphorylation sites in a protein sequence, with StBSL1 S489 predicted to be phosphorylated by a protein kinase A (PKA). In addition, this tool predicts 45 other serine residues that may be phosphorylated with varying probability, as well as 27 threonines and 3 tyrosines. Rather than mutating all of these, mass spectrometry may provide a useful starting point in narrowing these down by identifying phosphorylated residues. It could also be informative to compare differences in phosphorylated sites in the presence and absence of PiAVR2 to determine more about the role of StBSL1 activity in effector recognition. While this predicted phospho-site appears to have no effect on AVR2 recognition, it may still impact on BSL1 function at some level.

3.3 Effect of AVR2 and BSL1 on *P. infestans* virulence

PiAVR2 has been shown to interact with StBSL1, and StBSL1 has been shown to be required for recognition of effector AVR2 by resistance protein R2, potentially requiring BSL1 phosphatase activity. However, moving focus away from recognition and onto effector function, the fundamental question remains; Is StBSL1 a decoy, evolved to titrate PiAVR2 away from its true functional target, or does it play a role in pathogen virulence? The first step towards elucidating any function of StBSL1 in *P. infestans* virulence was to investigate whether this protein has a positive or negative effect on pathogen colonisation. *Agrobacterium tumefaciens* transient assays (ATTAs) were carried out in *Nicotiana benthamiana*, involving transient expression of StBSL1 and the mutant StBSL1 H648V, in addition to PiAVR2 and the variant effector AVR2-like. Leaves were subsequently inoculated with *P. infestans* sporangia and lesion size compared after seven days. Results can be seen in **Figure 3.6**.

The effector PiAVR2 was shown to significantly increase *P. infestans* pathogenicity. This complements previous work by Breen (2012) who showed that *P. infestans* transformants silenced in AVR2 expression have been shown to be greatly reduced in their virulence. These results suggest that the effector has an important role in promoting disease. PiAVR2-like, as discussed in the introduction, is a variant that evades R2-mediated recognition, thus isolates expressing this variant have a greater disease potential. AVR2-like was also shown to increase lesion size when transiently expressed in inoculated leaves, although not quite to the extent that AVR2 does. It may be that in evading recognition by R2, it has become slightly less efficient at its

function; a worthwhile trade-off in this case as pathogen isolates with this variant effector are currently thriving in the UK potato crop.

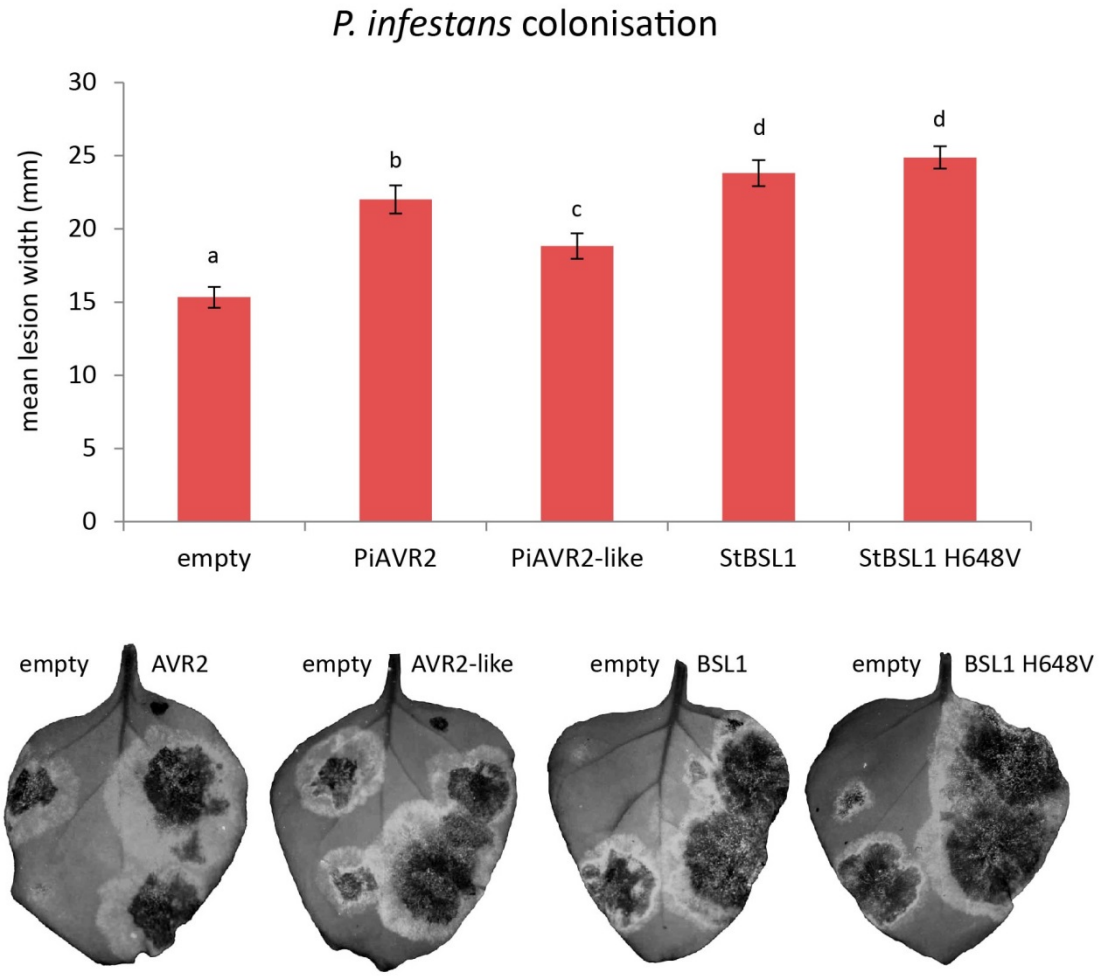

Figure 3.6 *Phytophthora infestans* colonisation with AVR2/BSL1 variants
Effector/target variants were transiently expressed in *Nicotiana benthamiana* followed by inoculation with P. infestans tdT. Lesions measured 7dpi. Error bars indicate SEM. a≠b,d p≤0.001, a≠c p≤0.01 c≠d p≤0.001 in one-way ANOVA (Holm-Sidak). Data combines a minimum of three biological replicates. Images taken under UV light and rendered in grayscale.

Surprisingly, when StBSL1 was transiently expressed, susceptibility also increased. If effector and target both boost *P. infestans* pathogenicity, it suggests that the function of PiAVR2 may be to increase the presence, or activity, of BSL1 in its host plant. Even more unexpected is the boost to pathogenicity seen with StBSL1 H648V. Despite the

wild-type and phosphatase-dead StBSL1 having opposite effects on the AVR2-R2 HR, their effect here was identical; a significant increase in *P. infestans* virulence. This is intriguing, and suggests that although phosphatase activity appears to be required for AVR2 recognition, it may not be required for the effector's function in virulence.

3.4 Function of AVR2 and BSL1 in pathogen virulence

The effector PiAVR2 and host target StBSL1 have now been shown to exert the same positive effect on *P. infestans* colonisation. To determine the basis of this increased virulence, the oomycete elicitin INFESTIN1 (INF1) was used in cell death assays. This elicitin is recognised in *N. benthamiana* as a PAMP, resulting in immune signalling and subsequent cell death in the form of the hypersensitive response. PiAVR2, AVR2-like, StBSL1 and StBSL1 H648V were transiently co-expressed with INF1 to investigate any effects on the immune response (see **Figure 3.7**).

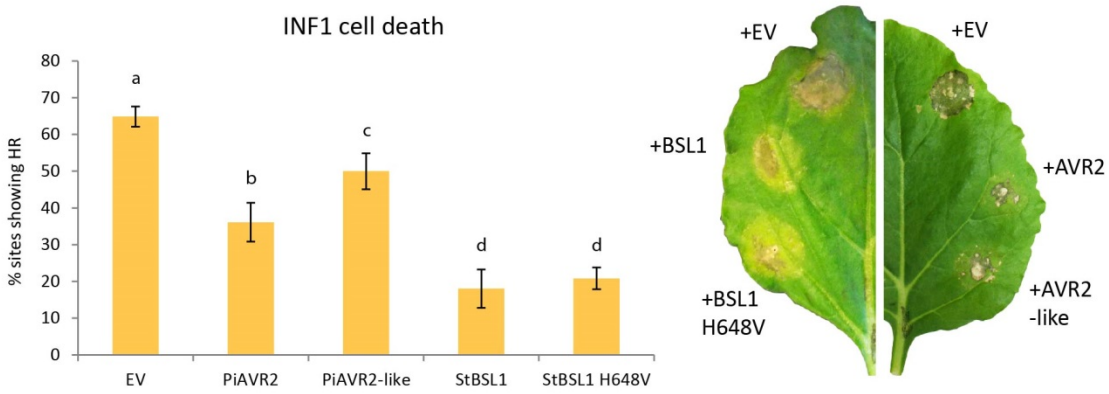

Figure 3.7 INF1 cell death with AVR2/BSL1 co-expression
Agrobacterium-mediated transient expression in *Nicotiana benthamiana*. Error bars indicate SEM. Lesions were scored 7dpi. a≠b,d p≤0.001, a≠c p≤0.05, c≠d p≤0.01 in one-way ANOVA (Holm-Sidak method). Data combines a minimum of three biological replicates.

The results show significant suppression of INF1 cell death by the presence of PiAVR2, suggesting the effectors function is to compromise aspects of plant immunity. This is a key role of pathogen effectors, and a crucial part of the 'zig-zag' model of plant defence (Jones and Dangl, 2006). Suppression of the immune response by an effector protein contributes to effector-triggered susceptibility (ETS). In the case of PiAVR2, this can be counter-acted by the presence of R2 in resistant plant lines and results in effector-triggered immunity (ETI). However, when R2 is not present in the host genome, the plant will remain susceptible and PiAVR2 will assist the pathogen in causing disease. The results also show suppression of INF1 cell death by the variant effector AVR2-like, although this suppression was not found to be as statistically significant as that seen with PiAVR2. This mirrors the results seen in the *P. infestans* colonisation assay; PiAVR2-like is not as effective at boosting pathogenicity, potentially because it is less effective at the suppression of plant immunity as seen here in the INF1 cell death assay (**Figure 3.7**).

Similarly to the pathogen effector, StBSL1 also suppressed the cell death elicited by INF1 in *Nicotiana benthamiana*. This again would explain its role in boosting *P. infestans* lesion size; a reduction in immune response will improve the pathogen's chance of successful colonisation. The StBSL1 H648V mutant is presumed non-functional due to a point mutation in the active site of the phosphatase domain, and as shown in section 3.3 it had an opposite effect to wild-type StBSL1 in PiAVR2 recognition – suppressing the HR, while the wild-type provided an increased HR. On the basis of this, it would be expected to perform differently in the INF1 cell death

assay, perhaps having no impact on cell death while the wild-type successfully suppressed it. This has not proven to be the case, with StBSL1 H648V not only successfully suppressing INF1 cell death, but achieving a level of suppression comparable to the wild-type. Although not in keeping with the AVR2-R2 HR result, this complements the *P. infestans* colonisation assay, where StBSL1 H648V increased lesion size to a similar extent as StBSL1 (**Figure 3.6**). It may indeed be that phosphatase activity of StBSL1 is unrelated to its role in immune suppression.

3.5 Linking the brassinosteroid pathway and PTI

Based on the discovery that StBSL1 can suppress INF1 cell death, it was next appropriate to determine whether this is linked to the role of StBSL1 in brassinosteroid pathway signalling, or if it alludes to an alternative function of BSL1 in potato. To investigate this, *Nicotiana benthamiana* plants were transiently silenced in the expression of *BAK1*, *BRI1*, and *BSL1* using Virus-Induced Gene Silencing (VIGS). TRV constructs were provided by Susan Breen and Eleanor Gilroy. As discussed in the introduction, BAK1 is not only the co-receptor for brassinosteroids in cohort with BRI1, but also functions as a co-receptor in the recognition of flagellin and elongation factor Ef-Tu, as well as recognition of INF1. These transiently silenced plants were then used for INF1 cell death assays to observe any impact on PAMP recognition and immune signalling (see **Figure 3.8**).

The results show a significant reduction in INF1 cell death when BAK1 is silenced. Because of BAK1's role in INF1 perception, this can be explained by a reduction in recognition of the elicitor. Conversely, when BRI1 was silenced, INF1 cell death was shown to increase. This could be attributed to the brassinosteroid pathways antagonistic effect on PTI. When BRI1 is silenced, there will be reduced perception of BR thus reduced pathway activity, potentially increasing the efficiency of the plant immune response to INF1. Silencing of BSL1 also had the same effect, with INF1 cell death accelerated. This complements the decreased cell death seen with StBSL1 over-expression. It also provides some evidence that BSL1 indeed functions positively in BR pathway signalling in the model Solanaceous plant *N. benthamiana*, with silencing achieving the same effect as silencing the BR receptor BRI1.

3.6 Effect of PiAVR2 on StBSL1 protein level

To examine any effect that PiAVR2 may have on StBSL1 protein level *in planta*, effector and target were co-expressed in *Nicotiana benthamiana* prior to protein extraction and western blot analysis (see **Figure 3.9**). This shows an increase in the level of StBSL1 when co-expressed with the effector, relative to co-expression with an empty vector control. Additionally, PiAVR2 is shown to have no effect on the protein StKHRBP, suggesting its action is specific to its target StBSL1. StKHRBP was recently shown by Wang *et al.* (2015) to be the target of another *P. infestans* effector, Pi04089. Pi04089 increases the stability of StKHRBP, and both effector and target increase *P. infestans*

pathogenicity in a similar manner to PiAVR2 and StBSL1. StKHRBP is regarded as a 'susceptibility factor' whose activity is beneficial for the pathogen.

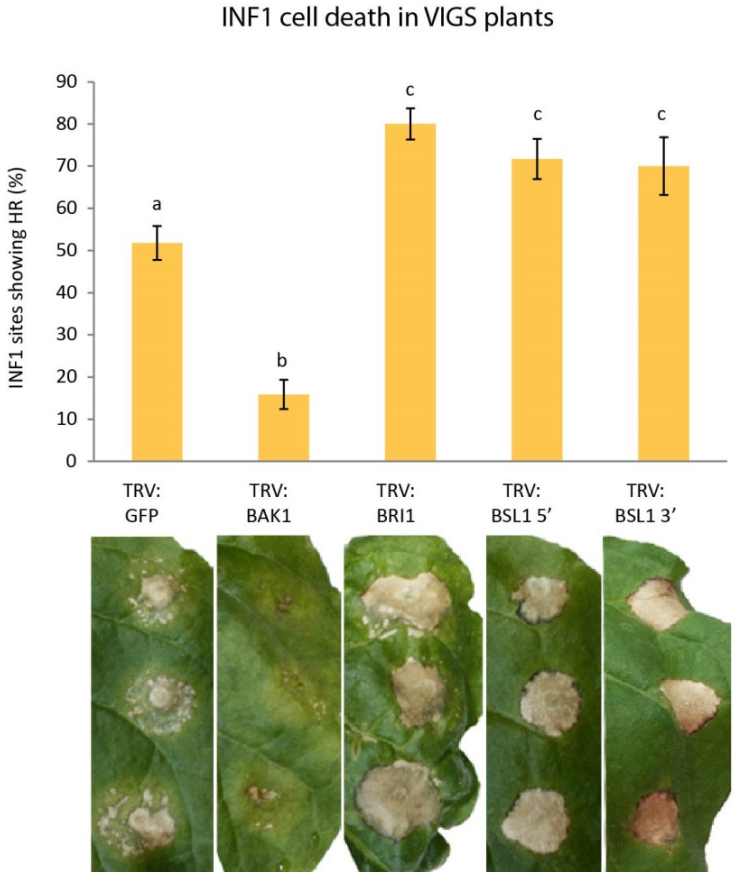

Figure 3.8 INF1 cell death in plants transiently silenced in BR pathway genes
Error bars indicate SEM, a≠b (p≤0.001) a≠c (p≤0.05) b≠c (p≤0.001) using one-way ANOVA (Holm-Sidak). Cell death scored at 5dpi. Data combines at least 3 biological replicates.

Figure 3.9b shows a separate biological replicate of StBSL1 +/- PiAVR2, with comparison to StBSL1 H648V. The phosphatase-dead mutant is also shown to be stabilised by PiAVR2, with a more intense band visible in the presence of the effector. Perhaps the most obvious explanation for this is that PiAVR2 can still interact with the

mutant StBSL1 H648V, despite the negative result found in the preliminary yeast-2-hybrid data. Alternatively, as already discussed, the mutant form may exist as dimers with endogenous BSL1 and be stabilised by association. Further analysis in the yeast-2-hybrid system or *in planta* by co-immunoprecipitation will be required to assess this. It would also be interesting to determine the mechanism by which PiAVR2 achieves the increase in StBSL1 protein level; directly by preventing turnover of existing StBSL1, or indirectly by increasing synthesis?

Notably, in Western blots for StBSL1 an additional band was frequently seen at approximately 70kDa (indicated by asterisks in **Figure 3.9**). This did not appear in control blots with the empty GFP-tagged vector, suggesting that it may be a fragment of GFP-StBSL1. If so, this would correspond to the GFP tag plus a region of the kelch domain, and could potentially mean that the phosphatase domain is cleaved from the rest of the protein. This band is also noticeably stronger in the presence of PiAVR2. **Figure 3.9** shows no corresponding 70kDa band for StBSL1 when co-expressed with an empty vector control, although this was variable and did appear, albeit faintly, without PiAVR2 in some cases.

This potential cleavage of the StBSL1 protein warranted further study, and a C-terminal fragment of StBSL1 was cloned. This consisted of the phosphatase domain plus a portion of the linker region between the kelch and phosphatase domains. This fragment is referred to as StBSL1Ct, and can be seen in the context of the full length protein in **Figure 3.11**. This fragment was used to investigate any differences between

the effect of StBSL1 full length and C-terminus both in AVR2 recognition, and in INF1-cell death (see **Figure 3.10**).

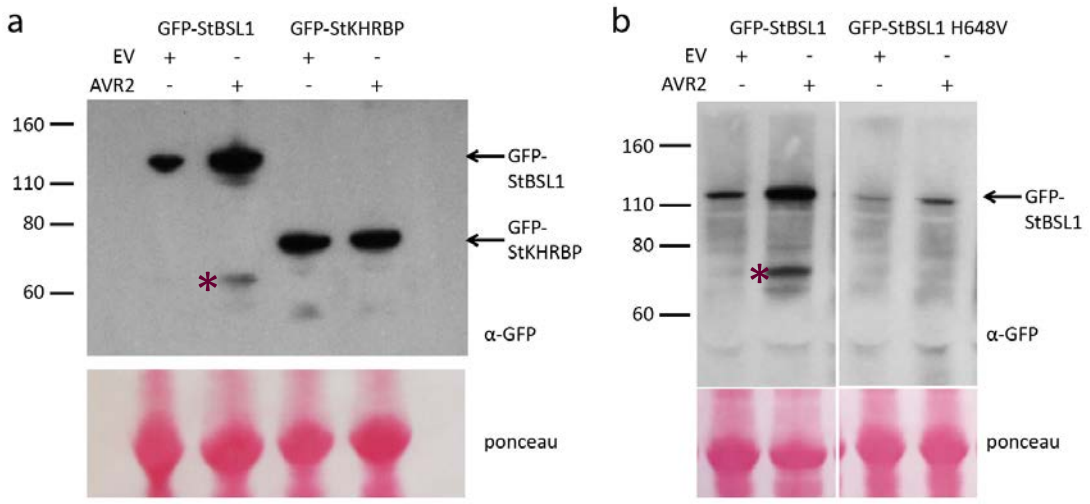

Figure 3.9 Increased stability of StBSL1 & StBSL1 H648V in the presence of PiAVR2
Transient expression of GFP-tagged constructs in *N. benthamiana* +/- pGRAB empty or PiAVR2. Leaf samples were taken 2dpi. a) StBSL1 compared to StKHRBP b) StBSL1 compared to StBSL1 H648V. Asterisk indicates additional band with fragment size equivalent to GFP-kelch domain.

These experiments show that the phosphatase domain of StBSL1 alone cannot suppress INF1 cell death, despite the full length protein doing so. This suggests the requirement of the kelch domain for function in this role. Conversely, the C-terminus of StBSL1 is capable of increasing AVR2 recognition similar to the full length protein, although this increase is slightly less effective than that with full length StBSL1. This suggests that the kelch repeat region is not required for the recognition of PiAVR2. These differences are interesting, and imply a dual role of StBSL1 in the plant.

In the functional characterisation of StBSU1, Mora-Garcia et al (2004) note that the kelch domain was not required for, and had no effect on, phosphatase activity, and the authors suggest that these domains behave independently. This appears to be the case for StBSL1 – the phosphatase domain alone can facilitate AVR2 recognition, but requires the active site to be intact. The kelch domain is required for the suppression of INF1 cell death by StBSL1, with no requirement of the active site. It may be that the kelch repeats alone would be enough to suppress INF1 cell death, but this is yet to be tested.

3.7 The BSL family in *Solanum tuberosum*

As described in Chapter 1, StBSL1 is a member of a family of phosphatases assumed to have overlapping function in brassinosteroid signalling. While four family members exist in Arabidopsis: AtBSU1, AtBSL1, AtBSL2 and AtBSL3, no orthologue of AtBSU1 can be identified in potato. The three family members present in the *Solanum tuberosum* genome are StBSL1, a putative orthologue of AtBSL1 (76% amino acid identity), and two genes that share 78-80% amino acid identity with both AtBSL2 and AtBSL3. These genes share 90% identity at the amino acid level, presumed to be paralogues arising from gene duplication after speciation, and are designated as StBSL2a and StBSL2b (Breen, 2012). An alignment of the amino acid sequences of StBSL2a and StBSL2b compared to StBSL1 can be seen in **Figure 3.11**.

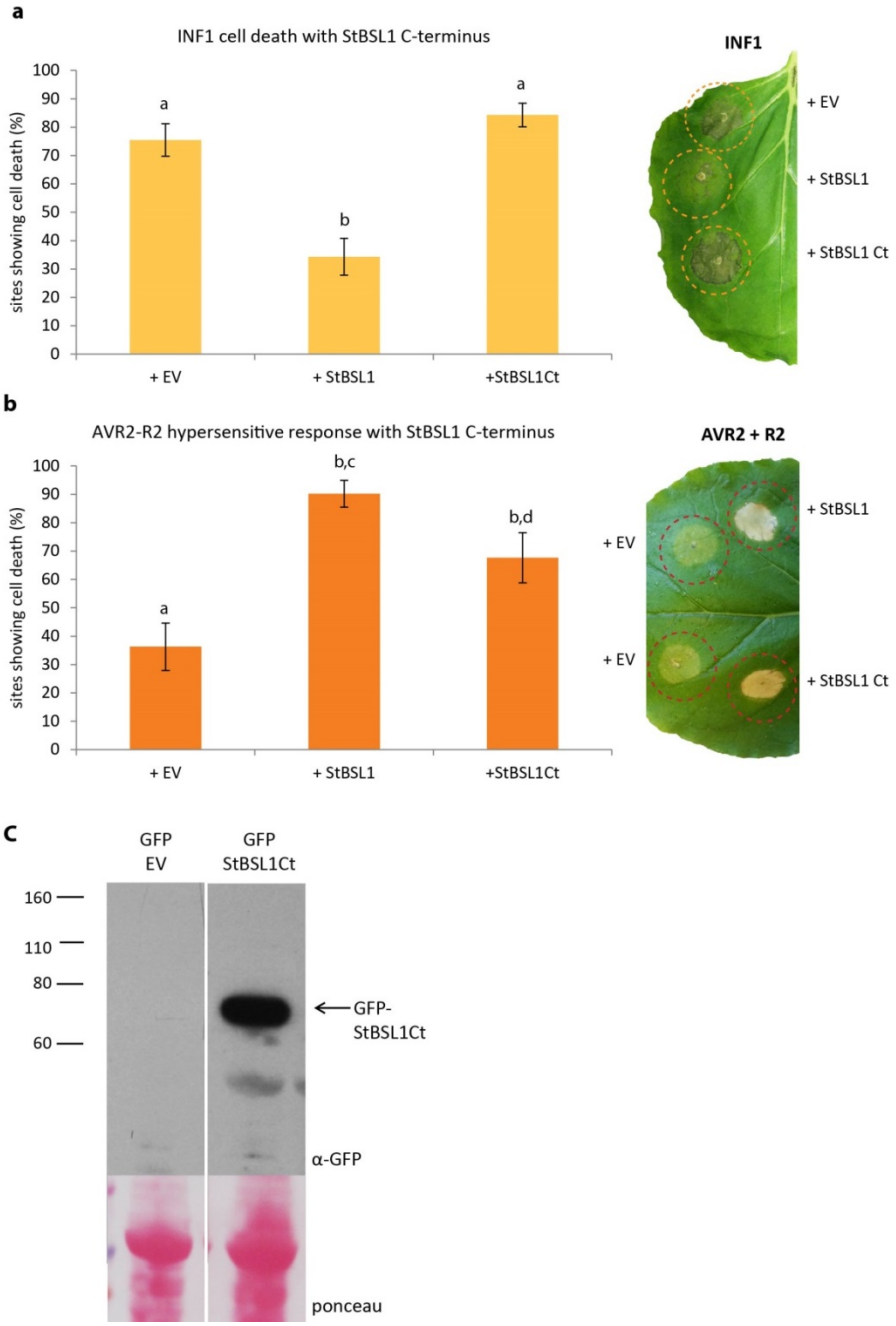

Figure 3.10 Effect of StBSL1 C-terminus on AVR2 recognition and INF1 cell death
Agrobacterium-mediated transient expression in *Nicotiana benthamiana*. **a.** INF1 cell death with StBSL1Ct compared to full-length StBSL1. Cell death scored at 7dpi. a≠b p≤0.001 in one-way ANOVA (Holm-Sidak method). **b**. AVR2-R2 HR with StBSL1Ct compared to full-length StBSL1. Cell death scored at 3dpi. a≠b p≤0.01, b≠c p≤0.05 in one-way ANOVA (Holm-Sidak method). **c**. western blot confirming stability of StBSL1Ct fragment. Leaf samples taken 2dpi.

The effector PiAVR2 has been shown to interact with both StBSL2a and 2b in yeast-2-hybrid analysis, as well as StBSL1 as previously discussed, indicating that the whole gene family may be relevant to pathogenicity. Additionally, Breen (2012) shows that StBSL2a and StBSL2b interact with each other. However, one important difference has been observed: whilst transient silencing of BSL1 in *N. benthamiana* has no developmental phenotype, silencing of BSL2a and 2b results in severely dwarfed growth. Runaway cell death is also evident when BSL2a/2b are silenced, which impedes experiments such as cell death assays on these plants. On the basis of these differences to StBSL1, it was considered important to investigate the function of these family members in PiAVR2 recognition and *P. infestans* virulence. Similarly to StBSL1 H648V, phosphatase-dead mutants of StBSL2a (H767V) and StBSL2b (H769V) were generated using site-directed mutagenesis. These were confirmed to be enzymatically inactive by Shaista Naqvi.

3.8 AVR2 stabilises StBSL2a and StBSL2b

When transiently co-expressed, PiAVR2 has already been shown to increase the relative protein level of StBSL1 (**section 3.6**). This was repeated with StBSL2a and StBSL2b to observe any effects, see **Figure 3.12**. This western blot shows StBSL2a, StBSL2b, and the active site mutant of each to be increased in stability by the presence of PiAVR2. This adds support to the finding that AVR2 interacts with both in yeast-2-hybrid analysis, and further implies that the mutation at the active site does not impede the interaction. Also notably there is an extra band present at around 70kDa –

this mirrors what is seen with StBSL1, and corresponds to the GFP tag plus kelch domain only. This family of PPKLs may have dual function, with the kelch repeats fulfilling a separate role from that of the catalytic domain. C-terminal regions of StBSL2a and StBSL2b, as well as the active site mutants H767V and H769V were cloned, but unfortunately not functionally characterised due to time restraints.

3.9 Effect of StBSL2a and StBSL2b on AVR2 recognition

With StBSL1 already shown to increase the AVR2-R2 hypersensitive response, and the active site mutant StBSL1 H648V shown to suppress it, the next step was to examine the effect of StBSL2a and StBSL2b on the AVR2-R2 hypersensitive response, see **Figure 3.13** and **3.14**. Given that StBSL1 has been shown to give a boost to the HR, and the active site StBSL1 H648V shown to suppress it, it was expected that overexpression of StBSL2a and StBSL2b would achieve the same thing. However this did not prove to be the case. **Figure 3.13** shows StBSL2a to have no effect on the HR, with StBSL2a H767V actually increasing cell death. However this increase should be interpreted cautiously, due to the experimental design used at this time. As shown in the leaf image, StBSL2a H767V was always co-infiltrated towards the leaf tip, and these sites are prone to showing an enhanced HR. Further experiments, including those already described with StBSL1, were designed differently on the basis of this, with infiltration sites rotated to minimise this location bias.

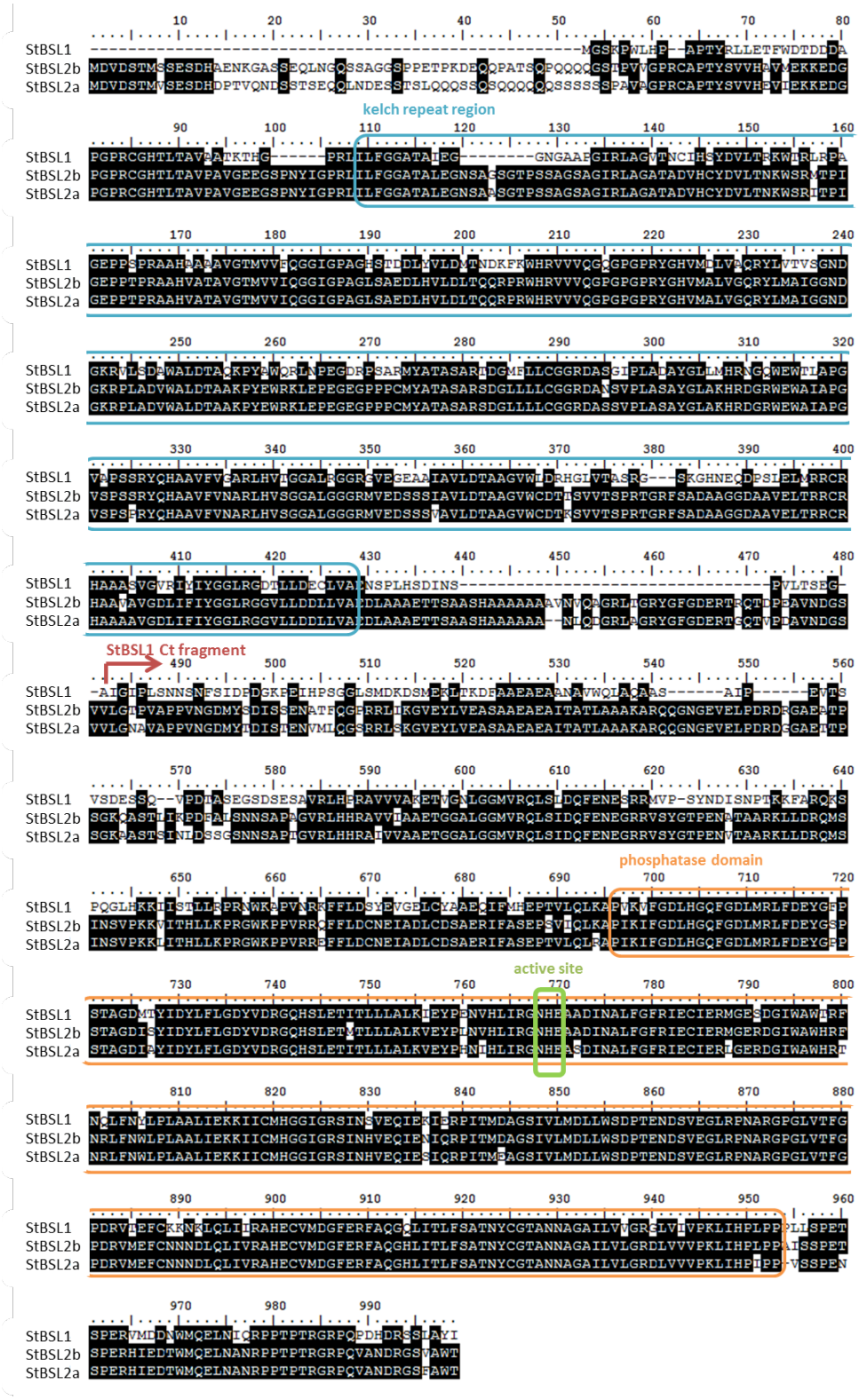

Figure 3.11 Amino acid alignment of the BSL family in potato
Blue box indicates kelch repeat region, orange indicates phosphatase domain, green box indicates active as predicted by Pfam. Red arrow indicates the region cloned as C-terminal fragment. Alignment generated by ClustalW in BioEdit.

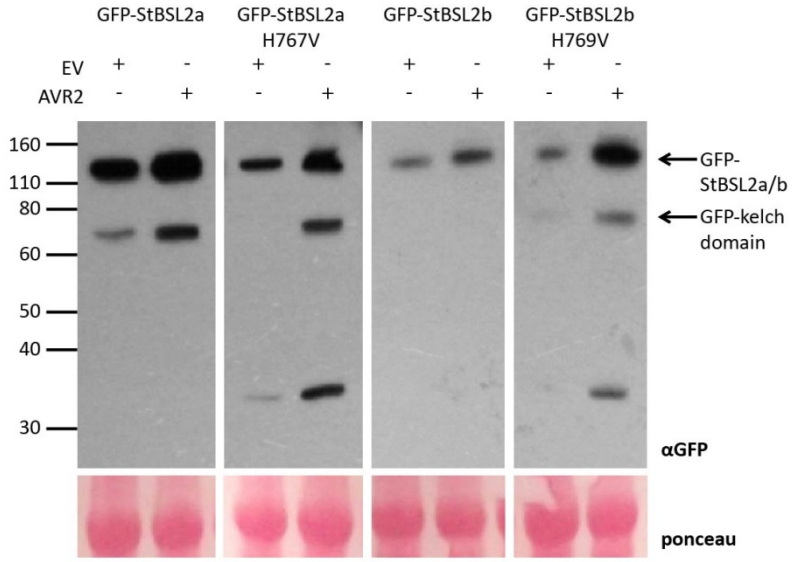

Figure 3.12 PiAVR2 increases relative protein level of StBSL2a and StBSL2b
Agrobacterium-mediated transient expression of pB7WGF2 StBSLs, plus pGRAB empty vector (EV) or PiAVR2. Leaf discs sampled at 2dpi.

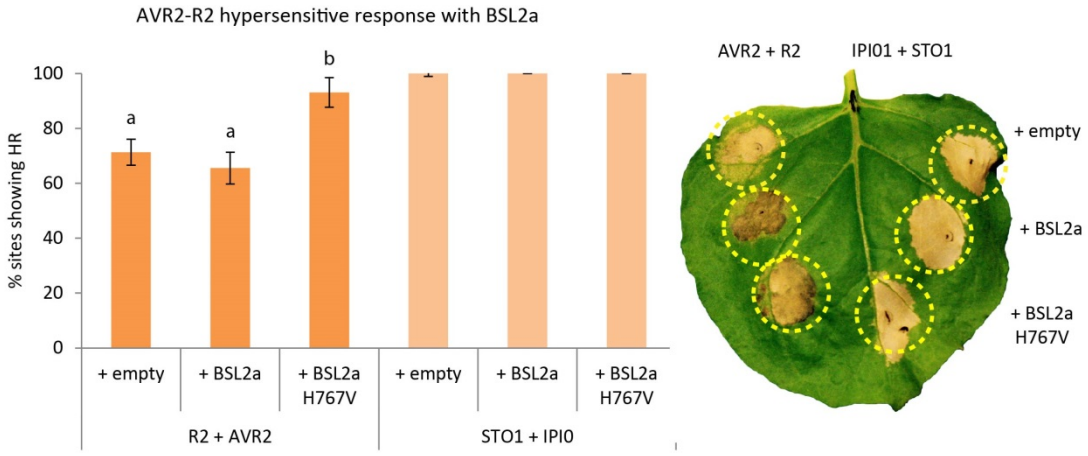

Figure 3.13 AVR2-R2 hypersensitive response with StBSL2a
Agrobacterium-mediated transient expression. Lesions were scored 7dpi. Error bars indicate SEM, with a≠b p≤0.05 in one-way ANOVA (Holm-Sidak method). Data combined across 3 biological replicates.

While StBSL2a appears to have no effect on AVR2 recognition, StBSL2b over-expression results in a significant suppression of the hypersensitive response, with the phosphatase-dead mutant not affecting the HR (see **Figure 3.14**). Although AVR2 interacts with all three of the BSLs, StBSL1 may be the only one with which R2 can associate. It may be that overexpression of StBSL2b titrates AVR2 away from BSL1, meaning that R2-mediated immune signalling is reduced. Alternatively, it might be that StBSL2b has a direct negative effect on StBSL1. If so, this appears to require phosphatase activity, as overexpression of StBSL2b H769V has no effect on the HR.

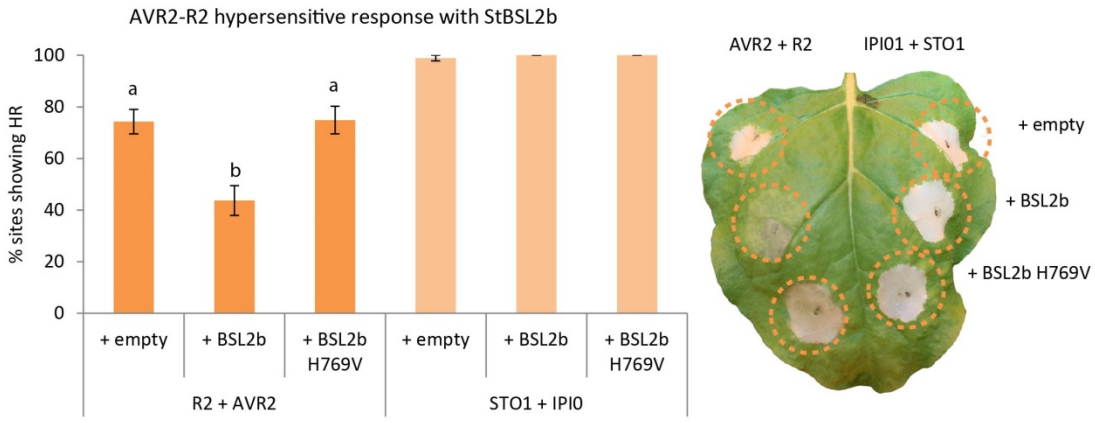

Figure 3.14 AVR2-R2 hypersensitive response with StBSL2b
Agrobacterium-mediated transient expression. Lesions were scored 7dpi. Error bars indicate SEM, with a≠b p≤0.001 in one-way ANOVA (Holm-Sidak method). Data combined across 5 biological replicates.

3.10 StBSL2a, StBSL2b and *P. infestans* virulence

To assess any role of StBSL2a and StBSL2b in pathogen virulence, these genes were silenced in *Nicotiana benthamiana* using VIGS (see phenotypes in **Figure 3.15**). Rather

than measuring lesion size, the variation in leaf size in these silenced plants made it more appropriate to measure simply whether an inoculated site gave rise to a sporulating lesion or not (**Figure 3.16**). The data shows a significant decrease in sporulating lesions on TRV:BSL2a and TRV:BSL2b plants compared to the GFP fragment control. This suggests that both BSL2a and 2b are required for *P. infestans* virulence, and is at odds with the previous results for the HR and INF1 cell death, which show BSL2a and BSL2b to have opposite effects. It may also be an artefact of the runaway cell death often seen in these plants; as a hemibiotroph, *P. infestans* requires living tissue to establish infection, and it may struggle to colonise the BSL2a and BSL2b silenced plants.

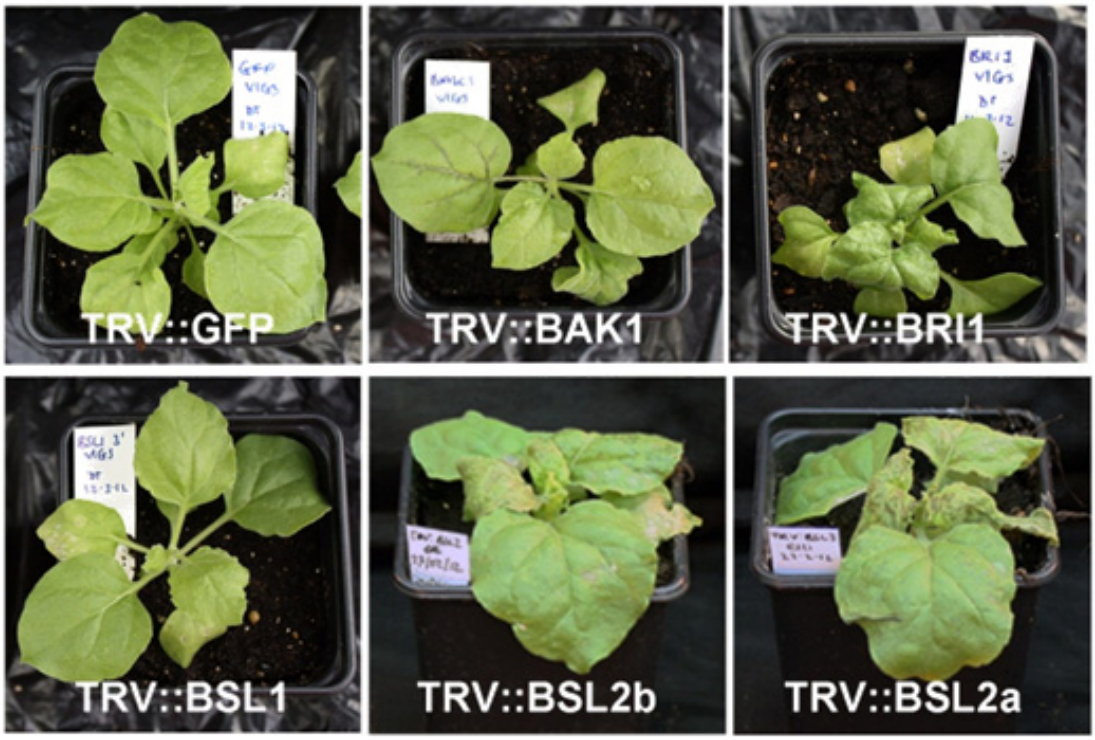

Figure 3.15 Phenotype of BR pathway VIGS in *Nicotiana benthamiana*
Nicotiana benthamiana plants transiently silenced in BAK1, BRI1, BSL1, BSL2a and BSL2b by virus-induced gene silencing (VIGS), compared to GFP control. Plants are shown approximately 2 weeks after infiltration with the virus construct.

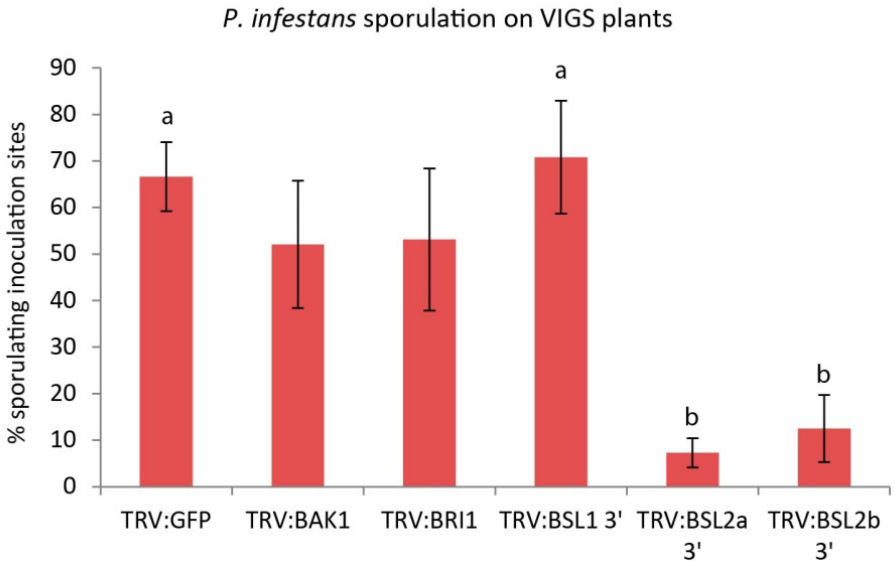

Figure 3.16 *P. infestans* sporulation on BR pathway VIGS plants
Nicotiana benthamiana plants transiently silenced for BAK1, BRI1, BSL1, BSL2a and BSL2b were inoculated with *P. infestans* sporangia suspension. Sites were scored on the basis of sporulation or non-sporulation 7dpi. a≠b $p \leq 0.05$ in one-way ANOVA (Holm-Sidak method). Data combines 4 biological replicates. The TRV:BSL2b construct reduces expression of both BSL2a and 2b so should be considered a double knock-out.

However it is worth noting at this point that the silencing construct for BSL2b is not entirely specific. While TRV:BSL2a reduces only BSL2a expression by 50%, TRV:BSL2b reduces both BSL2b and BSL2a expression to 50% (Breen 2012). This could mean that the phenotype seen in TRV:BSL2b plants is actually the result of off-target BSL2a silencing, and is not representative of a loss of BSL2b. If BSL2b is a negative regulator with effects opposite to StBSL1, its silencing would be expected to promote BR pathway activity, decrease PTI responses, and boost virulence which is the opposite of the result seen here. In these plants with both BSL2a and 2b reduced in expression, the loss of BSL2a could be expected to be dominant – if there is no positive regulation of the pathway (due to loss of BSL2a), then losing a negative regulator (BSL2b) will have

little or no effect. There is evidence of some difference between plants silenced with either of the two constructs; whilst TRV:BSL2a plants show the AVR2 HR to be reduced by 50%, there is only a 25% reduction seen in TRV:BSL2b plants (Breen 2012). This could in theory be because silencing of BSL2b relieves negative regulation of BSL1 and possibly the residual BSL2a in these plants, restoring AVR2 recognition to an extent. A specific silencing construct for BSL2b would be desirable to prove or disprove this theory, but the 90% sequence similarity between the two proteins makes this very challenging. It might be useful to utilise over-expression of StBSL2b in the TRV:BSL1 and TRV:BSL2a plants; when co-expressed with PiAVR2 and R2 it could potentially reduce the HR even further if it is indeed a negative regulator of the residual BSL1 or BSL2a.

No significant change in *P. infestans* sporulation can be seen in the other VIGS plants. There is a slight trend towards fewer sporulating lesions in TRV:BAK1 and TRV:BRI1 plants; this was a trend apparent in three of the four biological replicates but is not of statistical significance in the pooled data. BRI1 silencing might be expected to reduce pathogenicity; with reduced BR pathway activity increasing PTI responses, as seen in the increased INF1 cell death already shown. BAK1 silencing could conceivably affect *P. infestans* virulence in either direction. Its role in BR perception could mean reduced BR pathway activity, more PTI, thus reduced infection. Alternatively, its role in PAMP perception could mean less PTI when BAK1 is silenced, thus more infection. Notably, silencing StBSL1 has no impact on *P. infestans* sporulation. This is surprising given that StBSL1 overexpression boosts lesion size. It may be that in the absence of BSL1,

functional redundancy means that other family members can compensate. Complementary experiments, assessing *P. infestans* colonisation with transient over-expression of StBSL2a or 2b as opposed to silencing, would be a useful next step in identifying the function of these family members in pathogen virulence.

3.11 Effect of StBSL2a and StBSL2b on INF1-mediated cell death

To further examine the function of StBSL2a and StBSL2b and the active site mutants, their effect on INF1 cell death was assessed, used as a measure of PTI efficiency (see **Figure 3.17**).

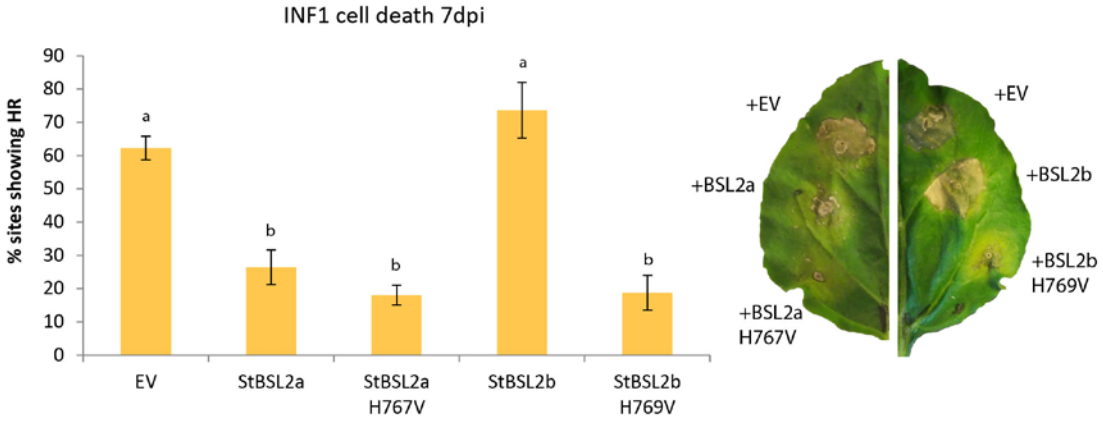

Figure 3.17 INF1 cell death with co-expression of StBSL2a and 2b
Agrobacterium-mediated transient expression. Cell death scored at 5dpi. Error bars indicate SEM. a≠b p≤0.001 in one way ANOVA (Holm-Sidak method). Data combines at least three biological replicates.

These results show that StBSL2a and the active site mutant StBSL2a H767V suppress INF1 cell death similarly to StBSL1 and StBSL1 H648V shown in section 3.5. This points to StBSL2a having overlapping function with StBSL1 in brassinosteroid pathway

signalling, thus inhibiting PTI. Surprisingly, StBSL2b does not behave in the same manner. There is no suppression of INF1 cell death with StBSL2b, on the contrary there actually appears to be an increased or accelerated response to INF1. This increase is not statistically significant at 5dpi when experimental replicates are combined, but it was clear from observing the experiment (and is visible in the photograph in **Figure 3.17**) that the cell death lesions with INF1 plus StBSL2b had progressed further than that with the empty vector control. The full extent of the HR (a pale, fully dessicated lesion) was reached more quickly. This is not reflected in the results in **Figure 3.17**, which are scored on the basis of a positive (>50%) or negative (<50%) cell death at a given infiltration site at 5dpi. It may be relevant to expand this scoring method in future, to highlight the strength of the HR as well as presence/absence.

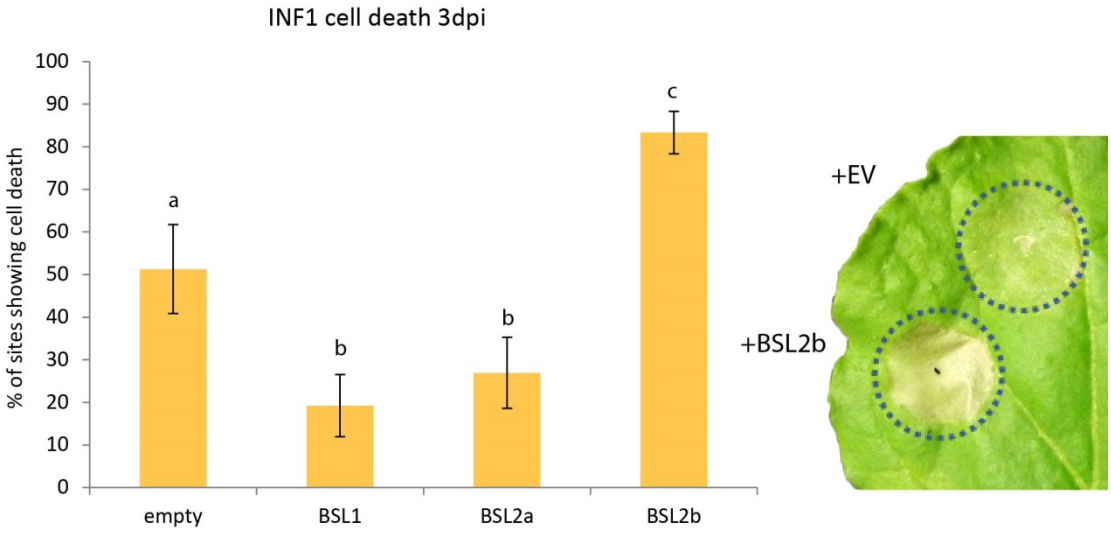

Figure 3.18 INF1 cell death with StBSL family at 3dpi
Agrobacterium-mediated transient expression in *Nicotiana benthamiana*. Lesions scored at 3dpi. a≠c p≤0.05, b≠c p≤0.001 in one-way ANOVA (Holm-Sidak). Data combined from at least 3 biological replicates.

Scoring lesions earlier, at 3 days post-infiltration, reveals the full extent of StBSL2bs effect on INF1 cell death (see **Figure 3.18**). This earlier scoring allows the visualisation of a clear boost to INF1 cell death with StBSL2b. Given the assumed overlap in function between BSL family members, this is an unexpected and intriguing result. It implies that the function of StBSL2b is not only different, but may be entirely opposite to that of StBSL1 and StBSL2a. Additionally, there is a clear difference in the impact of wild-type StBSL2b on INF1 cell death compared to the phosphatase-dead mutant. Whilst the wild-type protein accelerates INF1 cell death, the phosphatase-dead suppresses it (see **Figure 3.17**), suggesting that StBSL2b's ability to increase INF1 cell death is dependent on its phosphatase activity.

3.12 Discussion

The work outlined in this chapter aimed to determine whether StBSL1 has any role in pathogen virulence, or if it is a decoy evolved to titrate the effector away from its true target. The increased *P. infestans* colonisation, combined with decreased INF1 cell death when StBSL1 is over-expressed provide evidence that StBSL1 is not acting as a decoy. It presents an opportunity to the pathogen; a chance to suppress immunity indirectly by modifying StBSL1 activity. This beneficial effect on virulence identifies StBSL1 as a susceptibility (S) factor, defined as "plant genes that facilitate infection and support compatibility" (Van Schie and Takken, 2014). However it might be expected that silencing such a gene would have a negative effect on pathogen colonisation, and this did not appear to be the case, with *P. infestans* achieving equivalent levels of

sporulation on TRV:BSL1 plants and the control TRV:GFP plants. This points to PiAVR2 targeting more than just StBSL1, and possible functional redundancy between targets.

The observation that the AVR2-R2 hypersensitive response may require StSBL1 phosphatase activity is interesting – this could potentially be because R2 must be dephosphorylated in order to transduce an immune signal, or alternatively there could be another protein involved which StBSL1 acts upon. R2 has been shown to interact with StBSL1, only in the presence of PiAVR2 (Saunders *et al*., 2012), but it remains to be seen if any other proteins are present in this complex.

What is unexpected is that the active site mutation in StBSL1 H648V has no impact on the proteins ability to suppress INF1 cell death, or to boost colonisation by *P. infestans*. This may be because the mutation has disrupted AVR2 binding or R2 recruitment in some other manner. However, based on the experiments with the StBSL1 C-terminus alone, the explanation may be that phosphatase activity is not required for StBSL1s role in BR signalling/immune suppression at all. This PPKL protein may have a dual role, with the phosphatase domain functioning differently to the full length protein.

While the phosphatase domain alone can still facilitate AVR2 recognition, the removal of the kelch repeats renders StBSL1 ineffective at suppressing INF1 cell death. Also, although the phosphatase active site is required for AVR2 recognition, it is not required for immune suppression or increased pathogen virulence. Taken together, these observations support the hypothesis that the kelch domain alone may facilitate the signalling that leads to immune suppression. Further work using the isolated kelch

domain will be required to determine whether this is actually the case. A hypothetical model of the potential dual function of StBSL1 can be seen in **Figure 3.19**.

Dual function in proteins is widespread, and is referred to as 'moonlighting' (Huberts and Klei, 2010). A defining feature of these proteins is the independency of the functions; with directed mutations knocking out one function but leaving the other unchanged. Several examples of this have been characterised in plant growth and development. The phytosulfokine receptor AtPSKR1 is involved in plant growth responses; detecting PSK which incidentally is upregulated by brassinosteroids (Heyman *et al.*, 2013). AtPSKR1 signalling has also been shown to have inhibitive effects on PTI (Igarashi et al, 2012). This protein has dual function as both a kinase, and a guanylate cyclase (GC), with calcium concentration acting as a molecular switch between the two (Muleya *et al*., 2014). The GC catalytic domain is embedded, thus requires the protein to be re-folded in a different manner for activity. Notably AtBRI1, best known for its kinase activity in the recognition of BR, also exhibits an alternative function as a guanylate cyclase at a low level (Kwezi *et al*., 2007). Another example is the glucose sensor HXK1, characterised by Moore *et al*. (2003). With mutation to its hexokinase domain, AtHXK1 can be rendered catalytically inactive, losing its role in glucose metabolism yet still functioning in glucose signalling. Wong *et al*. (2013) hypothesise that dimerisation may also play a role in the switch between functions in moonlighting proteins. This could have relevance to StBSL1, with the finding that it interacts with itself in yeast-2-hybrid analysis.

The observation that StBSL1 may be cleaved in the plant suggests a role for the kelch domain and the phosphatase domain independently, which may differ when the full-length protein is intact. Separation of the two domains is likely to greatly change the proteins conformation, and may make other regions/active sites accessible when they were hidden previously. This line of enquiry suggests that there may be an extra layer of complexity in StBSL1s function. A key next step would be to confirm the identity of the additional protein band seen in Western blot, for which mass spectrometry may prove useful.

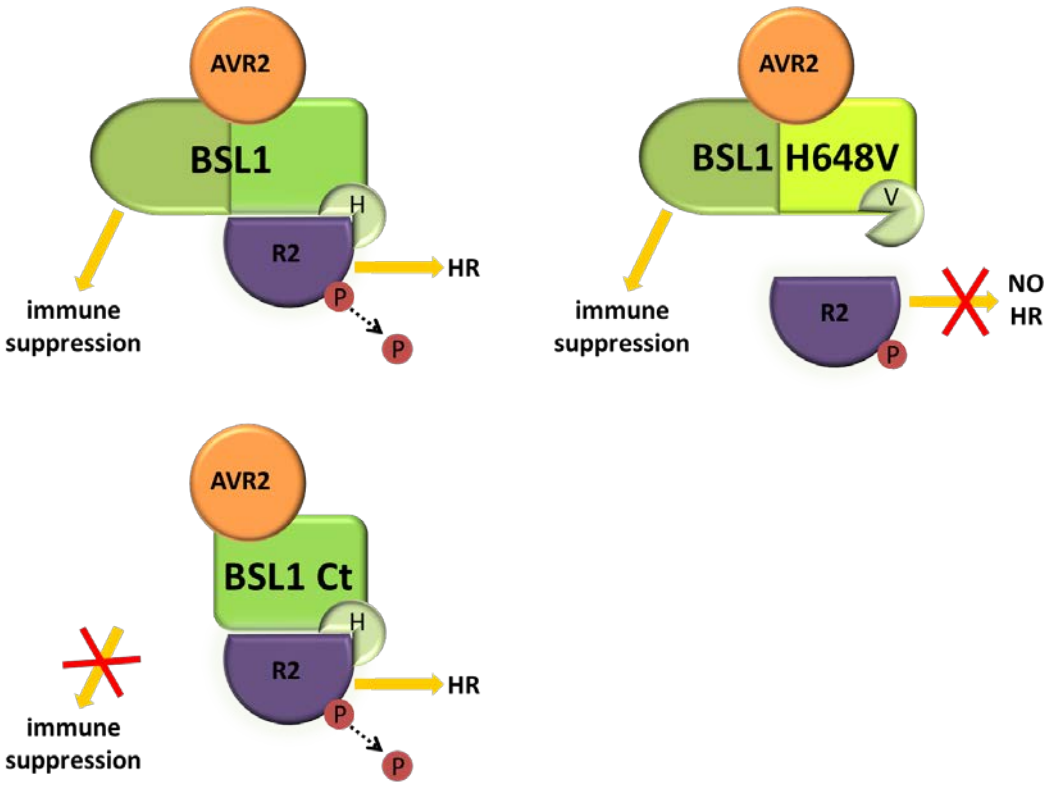

Figure 3.19 Hypothetical model of StBSL1 in the AVR2 hypersensitive response and immune suppression

A particularly interesting discovery in this work is that, contrary to assumption, the BSLs do not appear to have entirely overlapping function. Despite StBSL2a and StBSL2b sharing the highest sequence similarity, StBSL2a behaves more similarly to StBSL1; required for AVR2 recognition, and with a suppressive effect on INF1 cell death, suggesting a positive role in brassinosteroid signalling. StBSL2b overexpression has been shown to result in the opposite: suppressing PiAVR2 recognition, and increasing INF1 cell death. Based on this work, it can be hypothesised that StBSL2b may have a negative effect on StBSL1 and StBSL2a, and potentially a negative regulatory role in BR signalling. Breen (2012) identifies an interaction between StBSL2a and StBSL2b in yeast-2-hybrid, which supports the theory of StBSL2a as a putative substrate of StBSL2b. Whether StBSL2b can also interact with StBSL1 remains to be tested. In addition, the observation that StBSL2b can no longer accelerate INF1 cell death when mutated at the active site implies that phosphatase activity is required for this negative regulation. A model of the proposed interaction between the BSL family in potato can be seen in **Figure 3.20**.

While it seems unlikely that StBSL2a and StBSL2b, proteins with 90% sequence similarity, would have different functions, this is not unheard of. Small changes can potentially change the substrate, for example Alexander *et al.* (2009) describe the systematic mutation of a *Streptococcus* protein to alter its structure and function – with one amino acid substitution resulting in an entirely different folding conformation and change from an IgG binding to an albumin binding domain. Small changes can also change the function, as seen in Gal1p and Gal3p in Saccharomyces cerevisiae. These

proteins share 73% identity: Gal1p is a galactokinase, while Gal3p is a transcriptional inducer, with no galactokinase activity despite a galactokinase-like domain (Platt *et al*. 2000). The authors also describe that the addition of only two amino acids to Gal3p confers galactokinase activity. These examples reinforce the caution that is needed when inferring protein function from amino acid sequence, and assuming functional overlap between closely related family members. While it is a good starting point, it may not prove accurate. This appears to be true for the BSL family in potato, with StBSL2b having the opposite effect to StBSL1 in both the AVR2-R2 HR and effect on INF1 cell death.

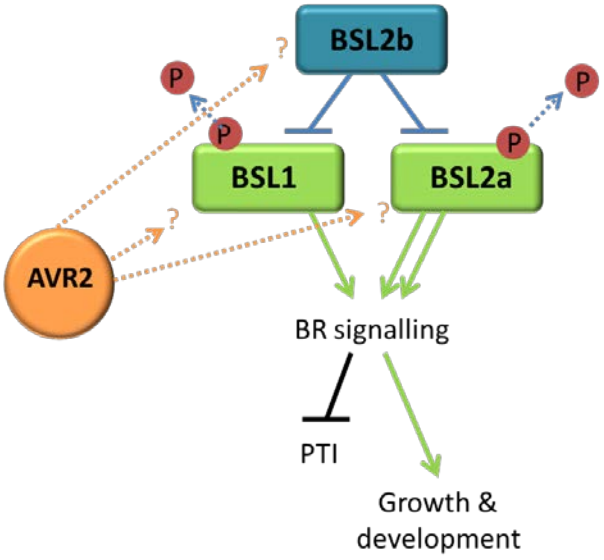

Figure 3.20 Proposed relationship between the BSL family members in potato

Crucially work by a colleague, Shaista Naqvi, has now shown that StBSL1, 2a and 2b do indeed have phosphatase activity, and that this is abolished in the active site mutants

designed and used in this work. Confirming the substrate of these phosphatases is another important next step. While AtBSU1 and AtBSL1 are both shown to interact with and dephosphorylate the kinase AtBIN2 (Kim *et al.* 2009), the same has not yet been shown in *Solanum tuberosum*. The predicted orthologue of BIN2 in potato is auto-active in the yeast-2-hybrid system (Breen, 2012), so future work will have to take advantage of alternative means of investigation such as co-immunoprecipitation, or bimolecular-fluorescence complementation. This could be complicated by the existence of family members, with overlapping functions thus degrees of redundancy, or possibly even different functions as seen in the BSL family in this study. AtBIN2 is a member of a family of 10 GSKs with overlapping function (Yan *et al*., 2009), and multi-gene famiilies appear to be a common theme in BR signalling components. Interestingly, Maselli *et al*. (2014) detect no effect of AtBSL1, AtBSL2 or AtBSL3 on BIN2 phosphorylation status, so it may be that their target is indeed a different protein, whether another member of the BIN2 family or another protein entirely. Qi *et al*. (2012) show that a BSL2 homolog in rice, GS3.1, interacts with and dephosphorylates Cyclin T1;3. Cyclin T1;3 is involved in the cell cycle, positively influencing grain size when phosphorylated. Overexpression of GS3.1 mutants with reduced phosphatase activity results in increased grain length, with these proposed to have a dominant negative effect on endogenous WT GS3.1. This points to a negative-regulatory role of BSL2 in rice growth, in support of the proposed role of StBSL2b as a negative regulator in brassinosteroid pathway signalling shown here. Further support is found by Maselli *et al*. (2014), who show that silencing AtBSL2 results in a phenotype more akin to to

BR-overactive mutants, with organ twisting, fusion and breakdown of symmetry, as opposed to the dwarf phenotypes associated with BR-knockouts.

The one observation that remains difficult to explain is the interaction between PiAVR2 and all three of the BSLs, despite StBSL1 and StBSL2a acting as apparent positive regulators of BR signalling while StBSL2b appears to do the opposite. This raises the question: which of the StBSLs is PiAVR2 actually targeting? While it is easy to see what the pathogen would gain from increasing levels of StBSL1 and StBSL2a (increasing BR pathway activity thus suppressing PTI) it is hard to hypothesise as to what it would gain from stabilising StBSL2b. Although, increased stability may not necessarily mean increased activity, and PiAVR2 binding to all three does not necessarily mean it will have an effect on all of them. Increased stability may be an artefact of decreased activity; potentially reducing endogenous protein turnover. There are two conceivable methods which *P. infestans* could use to increase BR pathway activity: over-activating a positive regulator, or de-activating a negative regulator. PiAVR2 could be targeting StBSL2b, and having a detrimental effect on its function. If StBSL2b is a negative regulator of StBSL1 and StBSL2a, PiAVR2 could be increasing the levels of these indirectly by removing the negative regulation. This would achieve the increased BR pathway activity and immune suppression.

Perhaps it is a matter of relevance in the plant tissue that is infected; maybe BSL2b is present at much lower levels than the others, so makes less of a contribution to the final outcome. The family members may be expressed differentially at particular times, during specific developmental transitions. Even if all are present at equivalent levels in

leaf tissue, splice variants, protein cleavage, and possible post-translational modifications may also play a role. The online resource eFP browser (Winter, 2007) allows the visualisation of gene expression levels in Arabidopsis in various organs, and it is clear from this that AtBSL1 is by far the most abundant BSL in leaf tissue, with absolute levels more than four times that of AtBSL2 or AtBSL3. Additionally, GUS fusions described by Maselli *et al.* (2014) show AtBSL1 to be strongly expressed in vegetative tissue, with AtBSL2 and AtBSL3 only expressed strongly in pollen. This could mean that PiAVR2s activity is primarily aimed at StBSL1, although these patterns may or may not reflect the BSL expression patterns in *S. tuberosum* and *N. benthamiana*.

The key to identifying the mode-of-action of PiAVR2 may lie in the interaction between the BSLs, both homo and hetero-dimerisation. A recent discovery by Kim *et al*. (2015) is that oligomerisation between these BSU1 family proteins potentiates BR signalling in Arabidopsis. Oligomerisation is shown to require a KKVI motif present in all family members, with mutation at these sites resulting in attenuated dephosphorylation of BIN2 as well as reduced affinity of AtBSU1 for AtBSK1. StBSLs contain this KKVI motif, with PiAVR2 also containing a similar region. Subcellular localisation of the BSLs may also prove to be relevant. Interestingly, co-localisation experiments described by Kim *et al*. (2015) show that hetero-oligomers between BSL family members are both nuclear and cytoplasmic, with the exception of those involving AtBSL1, which are excluded from the nucleus. It remains to be seen whether these localisations are recapitulated in potato. Preliminary co-immunoprecipitation results from Shaista Naqvi show that whilst all three StBSLs form strong homo-oligomers, hetero-oligomerisation

only occurs between StBSL2a and StBSL2b. The effect of PiAVR2 on this remains to be seen, but it may be that the effector disrupts the balance of these, or prevents oligomerisation entirely. For example, PiAVR2 could potentially interact with StBSL2b via its KKVI motif, preventing oligomerisation thus reducing function. Given the evidence that StBSL2b may have a negative regulatory role on the other BSLs, this reduced oligomerisation could provide the mechanism for PiAVR2s effects; relieving negative regulation of BR signalling, leading to increased growth and developmental outputs, and facilitating indirect suppression of the plants immune response.

3.13 Conclusions

This work identifies StBSL1 as a susceptibility factor in *P. infestans* infection; potentially targeted by the effector PiAVR2 in order to increase brassinosteroid pathway signalling and thus suppress the plants immune response. Also, the results add weight to the argument that this family of kelch phosphatases do not all function in the same manner. While results seen with StBSL1 and StBSL2a largely overlap and suggest a positive role in BR signalling, StBSL2b is shown to achieve the opposite; implying a negative regulatory role on the other BSLs and BR signalling in general. The role of StBSL1 in both the recognition of PiAVR2, and the suppression of immunity has been dissected, highlighting a potential dual role of StBSL1 whereby the phosphatase domain and kelch repeats function independently.

CHAPTER 4

Transcriptomic analysis of brassinosteroid-treated *Solanum tuberosum*

4.1 Introduction

Research to date has discovered many of the details of brassinosteroid (BR) pathway signalling, but it is by no means understood in its entirety. Layers of complexity, crosstalk and feedback between it and other signalling pathways mean that the commonly presented pathway structure is likely to be over-simplified, and the full extent of influence that brassinosteroid signalling has over the plants physiology is yet to be determined. In addition, the majority of published work focuses on the model plant *Arabidopsis thaliana*. This provides a valuable framework but care must be taken when applying the knowledge to other species, as differences as well as similarities are likely to exist. To begin to understand the impact of brassinosteroid signalling in the potato plant *Solanum tuberosum,* and to identify a set of BR marker genes relevant to this crop species, a transcriptomic approach was taken using microarray analysis. This examined differential gene expression induced by treatment with epibrassinolide (EBL), a highly active brassinosteroid, in a foliar spray at 50 µM. Two timepoints (3 hours and 24 hours) were selected to represent early and late responses, with BR-treated material compared to a mock-treated control at each timepoint. The microarray chips represented 60,000 transcripts, and were custom designed by the James Hutton Institute. Statistically significant changes in gene expression between treatments were determined by volcano filtering (t-test $p \leq 0.05$) with a cut-off of 2 fold up/down-regulation. Full details of experimental set up and data analysis can be found

in Materials and Methods **Section 2.10**. The microarray data in its entirety is included on CD-ROM as **Appendix 1**.

4.2 Experimental Aims

The aims of this chapter are:

- To examine brassinosteroid-induced transcriptomic changes in *Solanum tuberosum* plants
- To identify a set of BR marker genes that can be used to monitor brassinosteroid pathway activity in *S. tuberosum*
- To relate BR-regulated genes in potato to what is known about brassinosteroid signalling in the literature
- To compare differentially expressed genes in potato to those affected by BR in previously published work in other plant species

4.3 General findings

Microarray analysis of BR-treated *S. tuberosum* yielded a large number of differentially expressed transcripts: 254 significantly altered after 3 hours, and 2238 altered after 24 hours. Surprisingly, only 23 trancripts were found significantly changed at both timepoints, a relatively small overlap. Despite this, the heatmap in **Figure 4.1** shows that the majority of genes do respond in the same manner at both timepoints (ie. are up regulated at both 3h and 24h, or down-regulated at both 3h and 24h), however it appears that the extent of change is not strong enough to achieve statistical

significance at both. Overall, 2469 unique transcripts were detected as differentially expressed with BR treatment, representing over 4% of the total transcript number represented on the microarray chip.

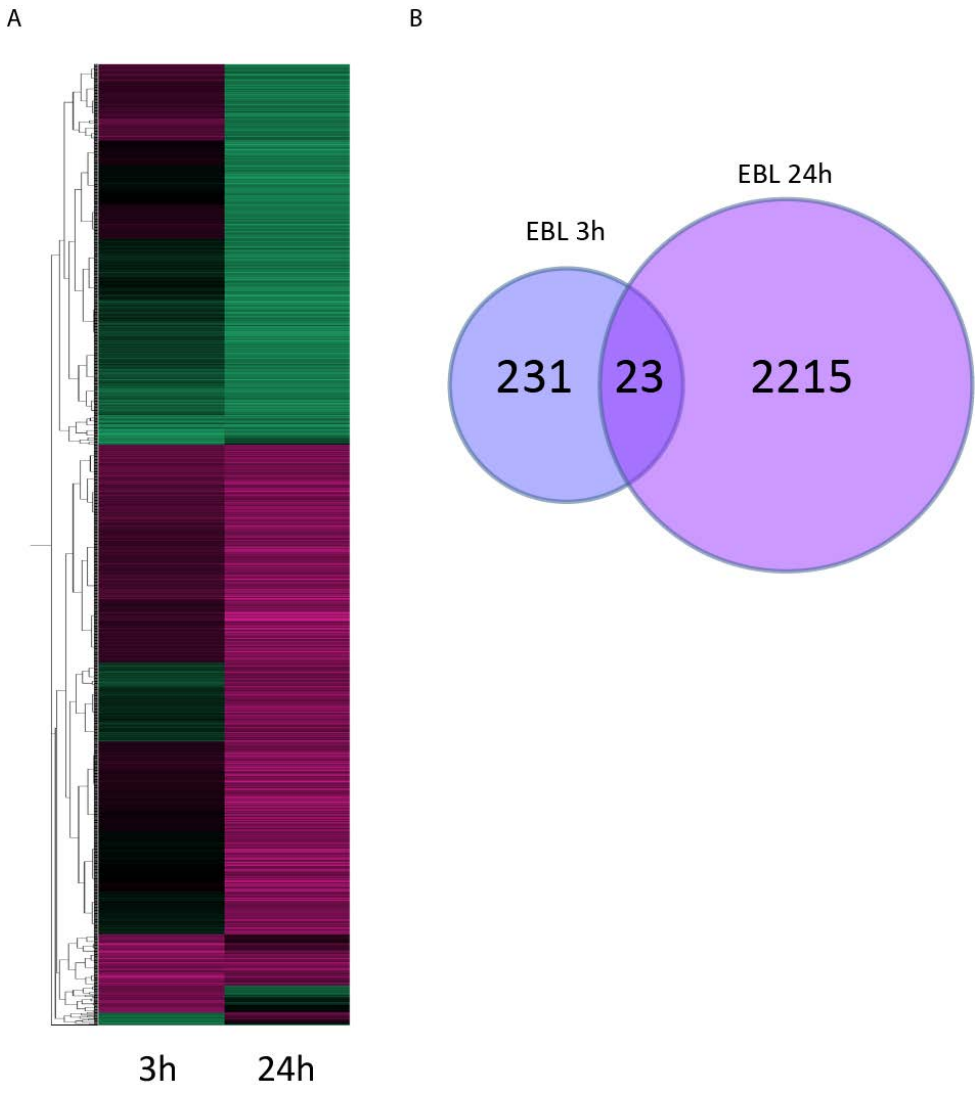

Figure 4.1 Heatmap of EBL-induced changes in *S. tuberosum* gene expression
A. Heatmap showing trend in differential expression with BR treatment at 3 hours and 24 hours. Upregulation or downregulation is represented by magenta or green respectively. B shows the relative contribution of each timepoint to the total number of transcripts differentially regulated by BR treatment.

The 3 hour dataset consists of a majority of significant transcripts being up-regulated (79%), whereas the 24 hour dataset is more balanced with 57% of genes up-regulated. The transcripts are each assigned to one of 35 functional categories based on a mapping of the potato genome in MapMan (Thimm *et al.,* 2004). The contribution of functional categories to the differentially regulated gene sets can be seen in **Figure 4.2**, separated by timepoint and by direction of regulation. Unfortunately, but perhaps as expected, the largest number of transcripts in each pie chart belongs to the category 'not assigned' or 'miscellaneous'. Excluding these, the three hour timepoint shows the highest number of transcripts assigned to the 'DNA' and 'RNA' categories. This may be the result of the wealth of transcriptional re-programming that occurs during hormone signalling. In keeping with this, the DNA and RNA categories are also well-represented in the 24h dataset, although are overtaken by transcripts assigned to the category 'protein', which may reflect the increased demand on protein regulation as a result of the increased transcription. Hormone metabolism is well-represented in the 24h dataset, which is to be expected, given that the plant will be not only dealing with the excess of brassinosteroid, but will also be integrating this signal into cross-talk with other hormone pathways. Also well-represented at the 24 hour timepoint are stress-related, and transport-related genes (showing both up and down-regulation), and a large number of photosynthetic and signalling genes showing upregulation. What is striking is the relatively low number of transcripts assigned to development, given that brassinosteroid is well-defined in its role as a positive regulator of growth and development. This may of course be down to category assignment; development is a broad function which will incorporate transcripts that could easily fit into one of the other functional categories. There is also inherent bias in these categories due to

differing numbers of transcripts assigned to each, as well as the fact that many genes have not been assigned. Over-representation analysis provides a more accurate means of determining which categories are truly enriched in the dataset, discussed in **Section 4.6**.

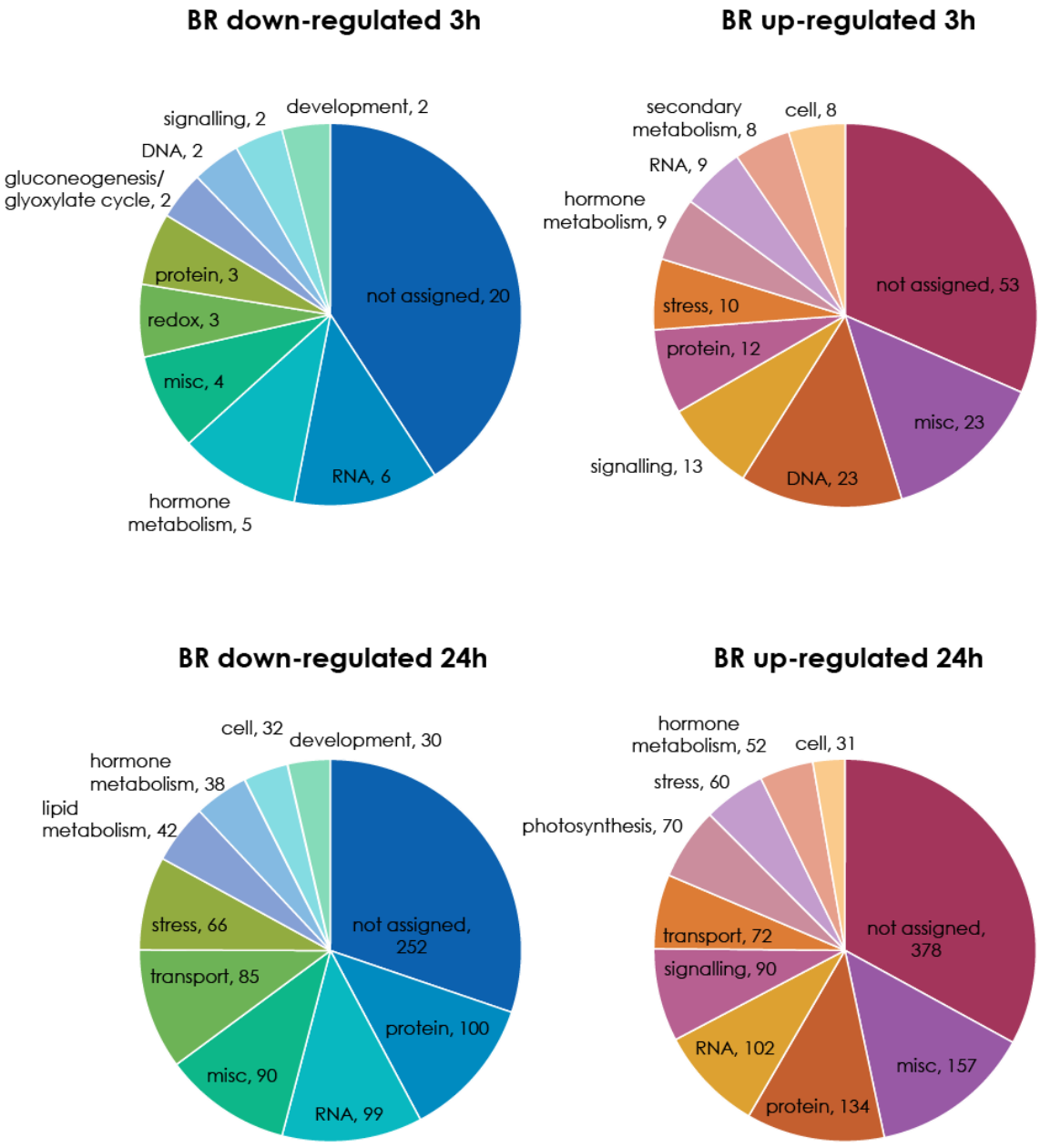

Figure 4.2 Functional categories of *Solanum tuberosum* transcripts affected by BR treatment at 3 hours and 24 hours
Numbers represent the number of transcripts significantly altered in expression for a given category. For clarity, only the top ten categories are represented.

4.4 Microarray validation and marker gene set

To validate the microarray data, five genes of interest were selected for analysis by qRT-PCR, using the same sample material as the microarray analysis. These genes were selected on the basis of links in the literature either specifically to brassinosteroid effects or to development in general. These genes can be seen in **Table 4.1** with corresponding microarray and qRT-PCR fold change data. To visualise the correlation between detection methods, fold-changes were $\log_2$ transformed and plotted on a scattergraph (see **Figure 4.3**). The co-efficient of determination (R^2 value) of 0.9688 as a result of linear regression indicates a very strong correlation between the microarray and qRT-PCR analysis. To further strengthen the data, qRT-PCR was used to examine changes in the expression of these genes in an independent biological replicate of BR treatment, conducted in the same manner. These results are plotted in relation to the microarray data in **Figure 4.4**, with an R^2 value of 0.9533. Together this gives confidence not only in the microarray data, but also suggests that the selected marker genes are a reliable indicator of active brassinosteroid pathway signalling in *S. tuberosum*.

Table 4.1 Selected BR-regulated marker genes for microarray validation

			fold-change with BR after 24h		
Gene number	**Gene name**	**UniRef based functional annotation**	Micro-array	qRT-PCR (original samples)	qRT-PCR (independent replicate)
PGSC0003DMG400016695	StCAB50	Chlorophyll a-b binding protein 50	13.368	8.301	5.193
PGSC0003DMG400000711	StBHLH7	BHLH7	13.035	4.936	6.845
PGSC0003DMG400003912	StP69F	P69F protein	9.366	5.321	3.131
PGSC0003DMG400016561	StSAUR67	Auxin-induced SAUR	2.561	2.682	2.520
PGSC0003DMG400021095	StGA2ox1	Gibberellin 2-oxidase 1	0.208	0.281	0.258

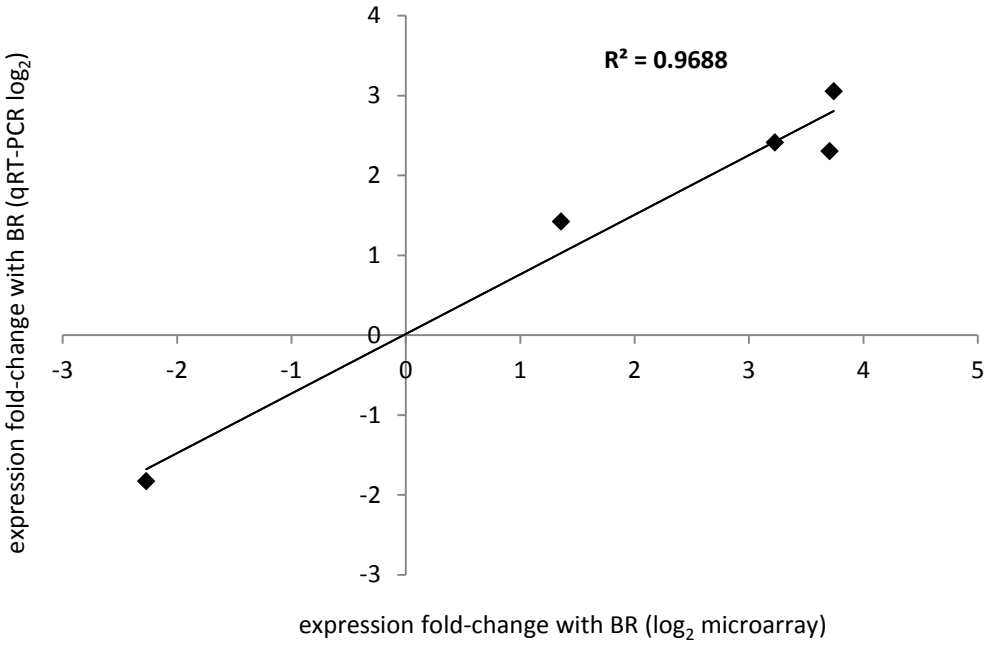

Figure 4.3 Correlation between microarray data and qRT-PCR data
qRT-PCR was used to measure changes in gene expression in the original RNA samples used for microarray analysis. Fold-change values are log2 transformed to allow symmetry of up and down-regulation. Data points are plotted against results from the microarray for each gene, with linear regression used to determine a coefficient of determination (R^2).

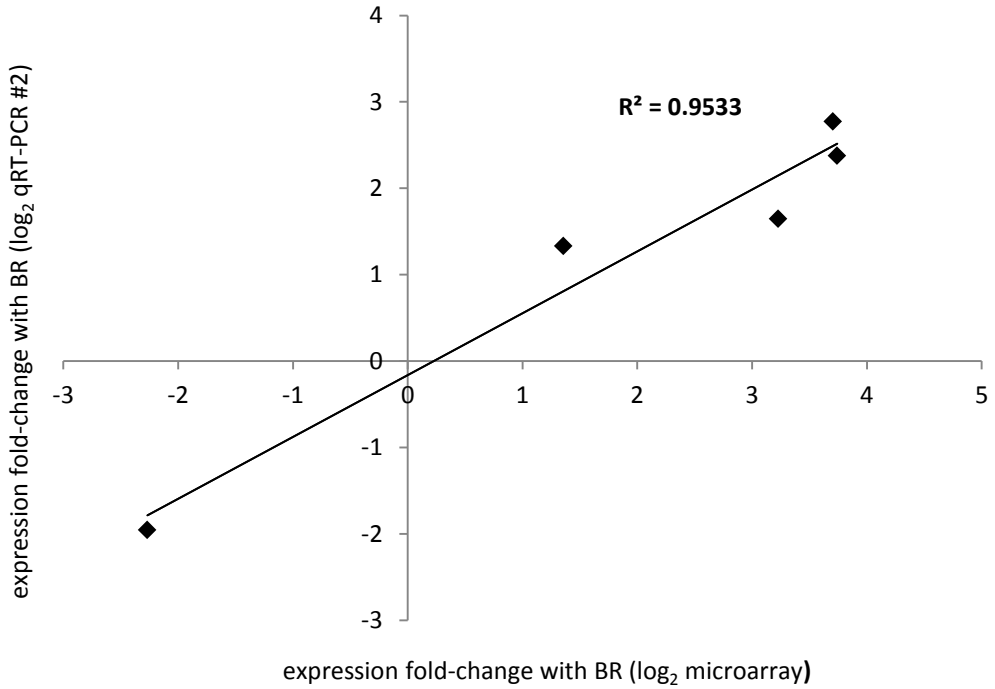

Figure 4.4 Correlation between microarray data and an independent replicate of BR treatment
qRT-PCR was used to measure changes in gene expression in an independent replicate of BR treatment on Solanum tuberosum. Fold-change values are log2 transformed to allow symmetry of up and down-regulation. Data points are plotted against results from the microarray for each gene, with linear regression used to determine a coefficient of determination (R^2).

The selected marker genes represent a variety of gene functions; photosynthesis, regulation of transcription, protein degradation, and hormone metabolism.

Chlorophyll a-b binding protein 50 (StCAB50), which functions as a light receptor in photosynthesis, is up-regulated 13-fold by EBL. This is one of 16 chlorophyll a-b binding proteins upregulated by brassinosteroid in this study. Photosynthesis-related genes have a strong presence in the data, and represent some of the most highly up-regulated genes. A total of 77 photosynthesis-related genes are differentially regulated at 24 hours post EBL treatment, only one of which is down-regulated. Photosynthesis in the context of these microarray results is discussed in **Section 4.6.12**.

Basic helix-loop-helix transcription factor BHLH7 is a member of a family of transcription factors, with several members shown to be up-regulated by brassinosteroids. Sequence analysis by BLASTp reveals the best matches in *Arabidopsis thaliana* to be AtCIB1 (At4g34530) and AtHBI1 (At2g18300), with AtHBI1 showing slightly higher amino acid identity at 41.95% compared to 40.5%. This identity exists in a highly conserved region of the protein. As discussed in **Chapter 1**, both HBI1 and CIB1 have been shown to suppress aspects of immunity in Arabidopsis, so this gene is of particular interest in the context of plant-pathogen interactions. BHLH7 will be referred to as StHBI1-like from this point, and is studied in further detail in **Chapter 6**.

15 BHLH transcription factors are differentially regulated 24 hours post-EBL, with a mixture of increased and decreased expression. Transcriptional regulators are well-represented in the data, with 166 showing differential expression. BHLH type transcription factors dominate, along with MYB domain, WRKY, homeobox, and AP2/EREBP transcription factors.

P69F protein (*StP69F*) is a subtilisin-like serine protease; upregulated more than 9-fold by EBL in the microarray data at 24 hours post-BR. Jorda *et al*. (2000) describe *P69F* in tomato as being specifically expressed in hydathodes, the water exuding organs found at vein tips in leaf margins. Given that this microarray utilised whole leaf tissue, and that *P69F* was one of the most highly upregulated transcripts, this seems unlikely to be the case in potato. The best match of this transcript in Arabidopsis is *ARA12*, a subtilase found at high levels in rapidly expanding cells (Hamilton *et al*., 2003), although this appears to be expressed in stems and siliques rather than leaves. Another is *STOMATAL DENSITY AND DIFFERENTIATION1* (*SDD1*) which is a subtilase shown to be involved in stomatal development. Von Groll *et al*. (2002) describe over-expression of *SDD1* as causing a decrease in stomatal density, and the arrested development of stomata. This is of particular interest in the context of the stomatal phenotype of 35S:AVR2 plants, discussed in **Chapter 5**.

Auxin-induced SAUR (*SMALL AUXIN UPREGULATED RNA*) is a member of the largest family of auxin-regulated genes with high sequence similarity. While auxin is well-characterised in its positive role in growth and development, the function of SAURS is largely unknown (Spartz *et al*., 2012). This particular *SAUR* most closely resembles *SAUR67* in Arabidopsis, and is one of 22 *SAURs* upregulated by brassinosteroid treatment in this study by 2 to 7-fold. Auxin associated genes differentially regulated by BR in this study are discussed in **Section 4.6.10**.

GIBERELLIN-2-OXIDASE1 (*StGA2ox1*) is an enzyme involved in the catalysis, or breakdown, of endogenous gibberellic acid (GA) in plants, and thus the regulation of plant growth (Lo *et al*., 2008). GA and brassinosteroids have been shown to act inter-dependently, and promote many of the same developmental responses (Bai *et al*.,

2012). The authors show that the promotion of cell elongation by GA requires brassinosteroid. The down-regulation of *GA2ox1* by BR in potato could potentially play a part in this: less GA would be broken down, therefore more would be available to act positively on plant growth and development. *GA2ox1* is down-regulated 5-fold by BR in this microarray.

4.5 Top 50 upregulated and downregulated gene lists

The 50 most up and down-regulated genes at 3 hours (early response) and 24 hours (late response) can be seen in **Tables 4.2** to **4.5**. These gene sets will be discussed in turn, with particular attention on genes of interest within these and their relation to brassinosteroid signalling and plant physiology in general.

4.5.1 Early response genes upregulated by BR

The top 50 BR upregulated genes can be seen in **Table 4.2**. The most highly up-regulated gene at 3 hours post EBL treatment is a cysteine protease inhibitor, one of three up-regulated at this timepoint. Cysteine proteases, enzymes which cleave their target peptides, are abundant proteins essential to life but can be damaging when overexpressed (Habib and Fazili, 2007). Cysteine protease inhibitors are involved in the regulation of these enzymes, and have been linked to the control of programmed cell death (PCD) (Solomon *et al*., 1999), with the balance between protease and inhibitor determining the outcome. Two stigma expressed proteins are also upregulated. These are trypsin inhibitors, with similarity to KTI1 in Arabidopsis. KTI1 is also linked to PCD, shown to inhibit the cell death expected from a fungal elicitor FB1, and to increase

Table 4.2 Early Response genes upregulated by BR (3h)

Gene ID	UniRef based putative functional annotation	Expression fold change with BR	MapMan functional category
PGSC0003DMG400010143	Cysteine protease inhibitor 1	11.78	not assigned
PGSC0003DMG400010137	Cysteine protease inhibitor 1	11.26	not assigned
PGSC0003DMG400025246	Histone H3.2	11.03	DNA (histone)
PGSC0003DMG400010136	Stigma expressed protein	10.69	stress (biotic, PR, trypsin inhib.)
PGSC0003DMG400001119	Histone H3.2	10.07	DNA (histone)
PGSC0003DMG400023522	Histone H4	9.81	DNA (histone)
PGSC0003DMG400010139	Cysteine protease inhibitor 1	9.25	not assigned
PGSC0003DMG400010146	Kunitz-type tuber invertase inhibitor	8.40	not assigned
PGSC0003DMG400023523	Histone H4	8.34	DNA (histone)
PGSC0003DMG401006333	Histone H3.2	7.21	DNA (histone)
PGSC0003DMG400025304	DNA-binding protein MNB1B	6.40	RNA (chromatin assembly)
PGSC0003DMG400024634	70 kDa subunit of replication protein A	6.08	DNA (synthesis/chromatin)
PGSC0003DMG400029085	Mta/sah nucleosidase	6.08	not assigned
PGSC0003DMG400010141	Stigma expressed protein	5.51	stress (biotic, PR, trypsin inhib.)
PGSC0003DMG400030349	CYP86A33 fatty acid omega-hydroxylase	4.98	misc (cytochrome P450)
PGSC0003DMG400016095	Anthocyanin acyltransferase	4.91	secondary metabolism
PGSC0003DMG400021869	Conserved gene of unknown function	4.67	not assigned
PGSC0003DMG400025261	Histone H3.2	4.61	DNA (histone)
PGSC0003DMG400012183	Endo-1,4-beta-glucanase	4.54	misc
PGSC0003DMG400012144	Conserved gene of unknown function	4.50	not assigned
PGSC0003DMG400026001	Uclacyanin-2	4.50	misc
PGSC0003DMG400010498	Photosystem II 5 kDa protein, chloroplast	4.37	not assigned
PGSC0003DMG400013633	Mini-chromosome maintenance protein MCM6	4.23	DNA (synthesis/chromatin)
PGSC0003DMG400010828	Organ-specific protein P4	4.18	not assigned
PGSC0003DMG400013439	Aspartic proteinase oryzasin-1	4.17	protein (degradation)
PGSC0003DMG400001948	Copalyl diphosphate synthase	4.15	secondary metabolism
PGSC0003DMG400001879	Annexin P38	4.10	cell (organisation)
PGSC0003DMG402007356	Thymidine kinase	4.06	nucleotide metabolism
PGSC0003DMG400002027	Cytoplasmic small heat shock protein class I	4.04	stress (biotic)
PGSC0003DMG400018930	Proteinase inhibitor I4, serpin	3.99	not assigned
PGSC0003DMG400029576	Polyphenol oxidase	3.94	not assigned
PGSC0003DMG400022836	Histone H3.2	3.93	DNA (histone)
PGSC0003DMG400007086	Mixed-lineage leukemia protein	3.90	RNA (SET-domain TF)
PGSC0003DMG400007020	DNA repair and recombination protein radA	3.86	DNA (repair)
PGSC0003DMG400020253	Ribonucleoside-diphosphate reductase	3.84	nucleotide metabolism
PGSC0003DMG400003626	Lactoylglutathione lyase	3.79	Biodegradation of Xenobiotics
PGSC0003DMG400016722	Glutathione S-transferase	3.78	misc
PGSC0003DMG400010892	Conserved gene of unknown function	3.75	DNA (synthesis/chromatin)
PGSC0003DMG400002943	Microsomal omega-6-desaturase	3.68	lipid metabolism (fatty acids)
PGSC0003DMG401027116	Laccase 90c	3.58	secondary metabolism
PGSC0003DMG400030514	Histone chaperone ASF1A	3.57	RNA (silencing group)
PGSC0003DMG400024748	Histone H2A.1	3.53	not assigned
PGSC0003DMG400029830	Glucan endo-1,3-beta-D-glucosidase	3.50	misc
PGSC0003DMG400010166	Anthocyanin permease	3.44	transport
PGSC0003DMG400000519	1,3-beta-glucan glucanohydrolase	3.43	misc
PGSC0003DMG400006233	Periaxin	3.43	not assigned
PGSC0003DMG401023841	Sterol desaturase	3.41	secondary metabolism (wax)
PGSC0003DMG400024397	RNase H family protein	3.39	not assigned
PGSC0003DMG400019098	Photosystem II 5 kDa protein, chloroplast	3.33	not assigned
PGSC0003DMG400019097	DNA repair protein XRCC2 homolog	3.31	DNA (repair)

susceptibility to Erwinia infection (Li *et al.* 2008). As discussed in **Chapter 1**, brassinosteroid signalling has links to PCD by means of its co-receptor BAK1, and the related BAK1-LIKE (BKK1). These are reported to maintain control over cell death (Kemmerling *et al*., 2007; He *et al*., 2007), with mutants displaying spreading cell-death lesions. This is reported to be brassinolide-independent, however the observation that StBSL2a silencing in *Nicotiana benthamiana* results in spontaneous cell death lesions (Breen, 2012), suggests that BR signalling does play a role.

Genes associated with DNA synthesis, chromatin and histones also feature strongly in the upregulated genes at 3 hours, and are discussed in more detail in **Section 4.6.1**.

4.5.2 Early response genes down-regulated by BR

The top 50 genes down-regulated by BR in potato can be seen in **Table 4.3**. The most down-regulated gene is ATL2N, a RING-H2 finger protein. RING-H2 finger proteins are so named because of their cysteine rich zinc-chelating motif, which appears in a large number of otherwise unrelated proteins (Jensen et al, 1998). StATLN2 is most similar in sequence to Arabidopsis At3g48030, annotated as being hypoxia-induced (TAIR, www.arabidopsis.org).

Also down-regulated is Callose synthase 8. Callose is a plant polysaccharide, present in the cell walls of higher plants, involved in roles such as cytokinesis, pollen development, and cell-cell movement of molecules (Chen *et al*., 2009). It is also widely recognised as being induced by stress, both biotic and abiotic, and is often used as a marker for PTI.

Callose deposition manifests as cell-wall thickenings called papillae at the site of infection, in which antimicrobial compounds can be deposited (Luna *et al*., 2011) and it provides a physical barrier to penetration. Belkhadir *et al*. (2012) show reduced PAMP-induced callose deposition in BRI1 and DWF4 over-expressing plants, evidence for the antagonism between BR signalling and PTI. Reduced callose synthase expression as a result of BR signalling may provide the mechanism for this observed decrease.

Interestingly, work by Nishmura *et al*. (2003) show that Arabidopsis with a mutated callose synthase gene GSL5, incapable of making pathogen-induced callose, is actually more resistant to pathogen attack by powdery mildew, and has hyperactive SA signalling which may be the means for the enhanced resistance. In contradiction to this, Ellinger *et al*. (2015) show even more effective resistance when this same callose synthase gene is overexpressed. This may be the result of completely different mechanisms; overexpression could potentially prevent pathogen entry completely, resulting in resistance, whereas knock-out lines would allow pathogen entry, but the hyperactive SA signalling would result in enhanced immune response. Luna *et al*. (2011) link callose deposition to ABA signalling, and report that pretreatment with ABA could increase, or repress, callose deposition dependent on environmental conditions. They also show that induction of callose deposition by two different elicitors, flg22 and chitosan, does not share the same signalling pathway. Based on this observed variation, the authors suggest caution is required when using callose deposition as a marker of PTI signalling.

Table 4.3 Early response genes downregulated by BR (3h)

Gene ID	UniRef based putative functional annotation	Expression fold change with BR	MapMan functional category
PGSC0003DMG400027719	RING-H2 finger protein ATL2N	0.18	protein (degradation,ubiquitin)
PGSC0003DMG400009073	Nodulin MtN3 family protein	0.25	development
PGSC0003DMG402004611	MYB transcription factor	0.26	RNA (MYB domain TF)
PGSC0003DMG400005526	Cytochrome P450	0.27	misc (cytochrome P450)
PGSC0003DMG400002835	DNA-binding protein	0.28	RNA (Homeobox TF)
PGSC0003DMG400023659	Thioredoxin	0.32	not assigned
PGSC0003DMG400021416	Conserved gene of unknown function	0.33	not assigned
PGSC0003DMG400027392	2-oxoglutarate-dependent dioxygenase	0.34	hormone metabolism (ethylene)
PGSC0003DMG400002341	Cytochrome P450	0.35	misc (cytochrome P450)
PGSC0003DMG400030657	Conserved gene of unknown function	0.36	not assigned
PGSC0003DMG400026535	Phytosulfokine peptide	0.37	not assigned
PGSC0003DMG400028302	Flavonol 4'-sulfotransferase	0.37	lipid metabolism (exotics)
PGSC0003DMG400028423	Conserved gene of unknown function	0.38	not assigned
PGSC0003DMG400032780	Conserved gene of unknown function	0.39	not assigned
PGSC0003DMG400016508	Conserved gene of unknown function	0.40	not assigned
PGSC0003DMG400026779	Twin lov protein	0.40	signalling (light)
PGSC0003DMG401007052	Callose synthase 8	0.40	minor CHO metabolism (callose)
PGSC0003DMG400006231	Gene of unknown function	0.41	not assigned
PGSC0003DMG401000567	Sugar transporter	0.41	transport (sugars)
PGSC0003DMG400011508	Conserved gene of unknown function	0.42	not assigned
PGSC0003DMG400028505	Conserved gene of unknown function	0.42	hormone metabolism (ethylene)
PGSC0003DMG400028381	Transcription factor	0.42	RNA (WRKY domain TF)
PGSC0003DMG400012020	Pectin methlyesterase inhibitor protein 1	0.43	misc
PGSC0003DMG400027729	Gene of unknown function	0.43	not assigned
PGSC0003DMG400040715	Gene of unknown function	0.44	not assigned
PGSC0003DMG400029727	Conserved gene of unknown function	0.44	not assigned
PGSC0003DMG401028902	ATP binding protein	0.45	signalling (DUF 26 RK)
PGSC0003DMG400043335	Gene of unknown function	0.45	not assigned
PGSC0003DMG400019832	Transport protein	0.45	protein
PGSC0003DMG400027224	Conserved gene of unknown function	0.45	not assigned
PGSC0003DMG400035835	ATP binding protein	0.45	hormone metabolism (cytokinin)
PGSC0003DMG400027577	Superoxide dismutase	0.45	redox
PGSC0003DMG400017338	Citrate synthase	0.46	gluconeogenese/ glyoxylate cycle
PGSC0003DMG400011287	DNA-3-methyladenine glycosylase	0.46	DNA (repair)
PGSC0003DMG400011287	DNA-3-methyladenine glycosylase	0.46	DNA (repair)
PGSC0003DMG400039968	Gibberellin 20-oxidase	0.47	not assigned
PGSC0003DMG400012263	Electron transporter	0.47	redox (glutaredoxins)
PGSC0003DMG400004036	ATP binding protein	0.47	protein (postranslational mod.)
PGSC0003DMG400001725	Pentatricopeptide repeat-containing	0.47	RNA (processing)
PGSC0003DMG400025556	WD-repeat protein	0.47	development
PGSC0003DMG400006237	Gamma-gliadin	0.48	not assigned
PGSC0003DMG400017378	Gene of unknown function	0.48	not assigned
PGSC0003DMG400000064	WRKY transcription factor 23	0.48	RNA (WRKY domain TF)
PGSC0003DMG400001937	Ethanol tolerance protein GEKO1	0.48	not assigned
PGSC0003DMG400013962	Amino acid transporter	0.48	transport
PGSC0003DMG400014823	XTH3	0.48	cell wall (modification)
PGSC0003DMG400015799	Conserved gene of unknown function	0.48	RNA (transcriptional regulation)
PGSC0003DMG400016663	Conserved gene of unknown function	0.49	not assigned
PGSC0003DMG400020118	Oxidoreductase, 2OG-Fe(II) oxygenase	0.50	not assigned
PGSC0003DMG400006073	Singapore isolate B (sub-type 7)	0.50	not assigned

4.5.3 Late response genes upregulated by BR

The most up-regulated transcripts at 24 hours post BR-treatment can be seen in **Table 4.4.** The most strongly upregulated are two pectinesterase genes, allocated to the functional category 'cell wall'. These enzymes are involved in the breakdown of pectin, and can contribute to cell wall loosening such as in fruit softening and cell elongation. Pilling *et al*. (2000) describe a transgenic potato plant overexpressing a pectinesterase gene, resulting in elongated stems in early development, and a reduction in tuber yield. Several 'major latex' transcripts are also upregulated, discussed in more detail in **Section 4.6.9**.

Four cytochrome P450 transcripts are upregulated by BR at 24 hours. These enzymes catalyse the addition of a single oxygen atom into a substrate, and account for around 1% of all genes in a plant genome (Mizutani *et al*., 2011). They are implicated in hormone synthesis and breakdown, secondary metabolism, defence compounds, signalling molecules and more, and are shown to be regulated by the circadian clock (Pan *et al*. 2009). Several cytochrome P450s have been linked to brassinosteroid biosynthesis, such as DWF4 (CYP90B1), CPD (CYP90A1), and ROT3 (CYP90C1). The potato transcripts upregulated by BR in this microarray are most similar to AtCYP75B1 involved in flavanol and anthocyanin biosynthesis (Pan *et al*. 2009), AtCYP78A9 linked to reproductive development (Sotelo-Silviera *et al*. 2013), and CYP71B34/B35 with no assigned function.

An interesting observation within the late response genes is the up-regulation of DWARF1 by BR treatment, expressed at a level 20-fold higher than untreated plants. This gene has been shown to convert 24-methylenecholesterol to campesterol in the sterol biosynthesis pathway, which feeds into that of brassinosteroid biosynthesis

Table 4.4 Late response genes upregulated by BR (24h)

Gene ID	UniRef based putative functional annotation	Expression fold change with BR	MapMan functional category
PGSC0003DMG401019255	Pectinesterase 3	70.25	cell wall
PGSC0003DMG400019256	Pectinesterase	48.87	cell wall
PGSC0003DMG400012100	Major latex	34.52	stress (abiotic)
PGSC0003DMG402027210	Glucosyltransferase	32.10	misc
PGSC0003DMG400008811	Major latex	32.01	stress (abiotic)
PGSC0003DMG400005526	Cytochrome P450	29.97	misc (cytochrome P450)
PGSC0003DMG400028725	Major latex	29.82	not assigned
PGSC0003DMG400012355	Conserved gene of unknown function	25.66	not assigned
PGSC0003DMG400028724	Major latex	22.48	stress (abiotic)
PGSC0003DMG400021142	DWARF1/DIMINUTO	20.05	hormone (BR synth/deg)
PGSC0003DMG400026346	F-box family protein	19.95	protein (degradation, ubiquitin)
PGSC0003DMG400004801	Conserved gene of unknown function	18.57	not assigned
PGSC0003DMG400026883	Gene of unknown function	17.54	not assigned
PGSC0003DMG400030784	Glutaredoxin family protein	17.06	redox
PGSC0003DMG400011740	SGA	16.47	misc
PGSC0003DMG400018925	Polyphenol oxidase B, chloroplastic	15.49	not assigned
PGSC0003DMG400016190	Anthocyanidine rhamnosyl-transferase	15.13	misc
PGSC0003DMG400026417	UPA22	15.08	signalling (light)
PGSC0003DMG400023514	Conserved gene of unknown function	14.76	not assigned
PGSC0003DMG402019255	Pectinesterase	14.64	cell wall
PGSC0003DMG400001144	Cytochrome P450 92B1	14.22	misc (cytochrome P450)
PGSC0003DMG400006721	Conserved gene of unknown function	14.19	not assigned
PGSC0003DMG400030483	Gene of unknown function	13.51	not assigned
PGSC0003DMG400014566	Transcription factor	13.44	RNA (CONSTANS-like, ZF family)
PGSC0003DMG400016695	Chlorophyll a-b binding protein 50	13.37	PS (lightreaction)
PGSC0003DMG400002859	Transcription regulator	13.32	RNA (bHLH TF)
PGSC0003DMG400013414	Chlorophyll a-b binding protein 3C	13.27	PS (lightreaction)
PGSC0003DMG400000711	Basic helix-loop-helix protein BHLH7	13.04	RNA (bHLH TF)
PGSC0003DMG400013415	Chlorophyll a-b binding protein 3C	12.81	PS (lightreaction)
PGSC0003DMG400018853	Sugar transporter	12.44	transport (sugars)
PGSC0003DMG400021838	Gene of unknown function	12.12	not assigned
PGSC0003DMG400013461	Chlorophyll a-b binding protein 3C	11.22	PS (lightreaction)
PGSC0003DMG400027187	Proline transporter 3	11.02	not assigned
PGSC0003DMG401002270	Mutt domain protein	10.84	not assigned
PGSC0003DMG400010525	Cytochrome P450	10.59	misc (cytochrome P450)
PGSC0003DMG400011548	Zinc finger DNA-binding protein	10.55	RNA (C2H2 ZF family)
PGSC0003DMG400028652	Gene of unknown function	10.47	misc.cytochrome P450
PGSC0003DMG400006841	SBT4B protein	10.43	protein (degradation)
PGSC0003DMG402002270	Mutt domain protein	10.17	nucleotide metabolism
PGSC0003DMG400015614	LATD/NIP	9.93	Transport (peptides/oligos)
PGSC0003DMG400009621	Cytochrome P450	9.84	misc (cytochrome P450)
PGSC0003DMG400041467	Zinc-finger protein	9.83	RNA (C2H2 ZF family)
PGSC0003DMG400025441	Conserved gene of unknown function	9.79	not assigned
PGSC0003DMG400002821	Conserved gene of unknown function	9.73	not assigned
PGSC0003DMG403002270	Mutt domain protein	9.70	not assigned
PGSC0003DMG402011794	ATP binding protein	9.66	protein (RLCK VII)
PGSC0003DMG400028789	Conserved gene of unknown function	9.54	not assigned
PGSC0003DMG400000584	Gene of unknown function	9.51	not assigned
PGSC0003DMG400003912	P69F protein	9.37	protein (degradation)
PGSC0003DMG400010232	Cysteine protease	9.31	protein (degradation)

(Choe *et al*., 1999). This is an unexpected result firstly because brassinosteroid biosynthesis has been shown to be affected by negative feedback: active BR signalling reduces the expression of many of the genes involved in BR biosynthesis, such as DWF4, CPD and ROT3 (Zhao and Li, 2012). It may be that sterol biosynthesis is subject to its own separate feedback mechanisms; perhaps DWF1 is up-regulated to attempt to compensate for the downregulation of downstream steps. DWF1 specifically has previously been shown by Tanaka *et al*. (2005) to be unaffected by exogenous BR in Arabidopsis, making the strong up-regulation seen in this study even more surprising. It may of course be unrelated to brassinosteroid signalling; campesterol is an active compound in its own right and is required for control of membrane fluidity (Piironen *et al*., 2000), which has been linked to perception of environmental signals such as temperature (Mikami *et al*., 2003).

This gene set also contains the genes selected in section 4.1 as markers of BR signalling in potato; BHLH7 (renamed as StHBI1-like), StP69F and StCAB50, as already discussed in **Section 4.4**.

4.5.4 Late response genes downregulated by BR

The fifty transcripts most down-regulated by BR can be seen in **Table 4.5**. The most down-regulated transcript, PGSC0003DMG400014212, is annotated as a heat shock protein and is one of over fifty heat shock proteins down-regulated in this microarray (and one of 9 in the top 50 most down-regulated transcripts). These are associated with abiotic stress, and are discussed in more detail in **Section 4.6.9**, with potential

links to the antagonism between BR-mediated growth and ABA-mediated stress responses.

Table 4.5 Late response genes downregulated by BR (24h)

Gene ID	UniRef based putative functional annotation	Expression fold change with BR	MapMan functional category
PGSC0003DMG400014212	Heat shock protein	0.03	not assigned
PGSC0003DMG400003530	ABA and environmental stress-inducible TAS14	0.04	not assigned
PGSC0003DMG400002895	Sucrose synthase	0.05	major CHO metabolism
PGSC0003DMG400014863	Phenylacetaldehyde synthase	0.05	secondary metabolism
PGSC0003DMG400028543	Gene of unknown function	0.05	not assigned
PGSC0003DMG400008187	Class II small heat shock protein Le-HSP17.6	0.05	stress (abiotic, heat)
PGSC0003DMG400012838	Non-specific lipid-transfer protein	0.05	lipid metabolism
PGSC0003DMG400001518	Conserved gene of unknown function	0.06	not assigned
PGSC0003DMG400046998	S-adenosylmethionine-dependent methyltransferase	0.06	development
PGSC0003DMG400005269	Glucose-6-phosphate/phosphate translocator 2	0.06	transport
PGSC0003DMG402007944	Gene of unknown function	0.06	not assigned
PGSC0003DMG400010128	Serine protease inhibitor 7	0.07	not assigned
PGSC0003DMG400022304	Extracellular ligand-gated ion channel	0.07	RNA (AP2/EREBP family)
PGSC0003DMG400028622	Acyl-protein thioesterase	0.07	lipid metabolism
PGSC0003DMG400011438	Protein LE25	0.08	development
PGSC0003DMG400006578	Lipoprotein	0.08	not assigned
PGSC0003DMG400007233	CXE carboxylesterase	0.09	Biodegradation of Xenobiotics
PGSC0003DMG400014293	Low-temperature-induced 65 kDa protein	0.09	stress (abiotic, cold)
PGSC0003DMG400028221	Tropinone reductase I	0.10	misc (nitrilases)
PGSC0003DMG400030339	17.6 kD class I small heat shock protein	0.10	stress (abiotic, heat)
PGSC0003DMG400012837	Non-specific lipid-transfer protein	0.10	lipid metabolism
PGSC0003DMG400009112	Protein phosphatase 2C 8	0.10	protein (postranslational mod.)
PGSC0003DMG400002463	Glutathione s-transferase	0.11	misc
PGSC0003DMG400002291	Triacylglycerol lipase	0.11	lipid metabolism
PGSC0003DMG400010388	DNA binding protein	0.11	RNA (bHLH family TF)
PGSC0003DMG400000248	HB1	0.11	RNA (Homeobox TF family)
PGSC0003DMG402004500	Glycosyltransferase 1	0.12	misc
PGSC0003DMG400019956	Glutathione s-transferase	0.12	misc
PGSC0003DMG400005573	Heat shock protein	0.12	stress (abiotic, heat)
PGSC0003DMG400013632	Conserved gene of unknown function	0.12	not assigned
PGSC0003DMG400016984	Alcohol NADP+ oxidoreductase	0.12	secondary metabolism
PGSC0003DMG400009512	Kunitz-type proteinase inhibitor	0.13	not assigned
PGSC0003DMG400000444	Heat shock cognate 70 kDa protein	0.13	stress (abiotic, heat)
PGSC0003DMG400004670	Xyloglucan endo-transglycosylase	0.13	cell wall (modification)
PGSC0003DMG400011632	Chloroplast small heat shock protein class I	0.13	stress (abiotic, heat)
PGSC0003DMG400012623	NOI	0.13	signalling (sugar and nutrients)
PGSC0003DMG400020131	Non-specific lipid-transfer protein	0.13	misc
PGSC0003DMG400020605	MLO1	0.14	stress (biotic, MLO-like)
PGSC0003DMG400032793	Heat stress transcription factor HSFA9	0.14	not assigned
PGSC0003DMG400028021	Short chain alcohol dehydrogenase	0.14	misc
PGSC0003DMG400025395	Nitrate transporter	0.14	transport
PGSC0003DMG400011628	Chloroplast small heat shock protein class I	0.14	stress (abiotic, heat)
PGSC0003DMG400021877	Xyloglucan endo-transglycosylase	0.14	cell wall (modification)
PGSC0003DMG402028907	Heat shock protein 90	0.14	stress (abiotic, heat)
PGSC0003DMG400010430	Conserved gene of unknown function	0.14	cell (organisation)
PGSC0003DMG400012577	Erg-1	0.14	signalling (sugar and nutrients)
PGSC0003DMG400002987	DNAJ protein	0.15	stress (abiotic, heat)
PGSC0003DMG400004169	Gene of unknown function	0.15	not assigned
PGSC0003DMG400000110	Wax synthase	0.15	not assigned
PGSC0003DMG400024818	Nodulin MtN3 family protein	0.15	development

The second most down-regulated transcript is annotated as ABA and environmental stress-inducible TAS14 – a 'dehydrin' associated with increased drought and salinity tolerance when over-expressed in tomato (Munoz-Mayer *et al.,* 2012). This again is in keeping with antagonism between BR and ABA signalling.

A nodulin MtN3 family gene is present in the top 50 most down-regulated genes. This is one of 6 nodulin transcripts found to be BR-regulated in this microarray, 5 of which are down-regulated at both timepoints (although only significantly so at 24 hours), and one which is significantly down-regulated at 3 hours but significantly up-regulated at 24 hours. Nodulins were originally identified as being involved in Rhizobium-induced nodulation, but homologues exist in non-nodulating plants, suggestive of a broader function. The MtN3 family is also referred to as the SWEET family, and has been linked to sugar transport and that of aluminium and copper (Denance *et al*., 2013). Interestingly, SWEET genes have been shown to be a pathogen effector target, with five conferring susceptibility to *Xanthomonas* infection in rice (Streubel *et al*., 2013). The upregulation of these genes is presumed to play a role in pathogen nutrition; increasing phloem unloading into the apoplast, thus increasing carbon availability for the pathogen (Lapin and Van der Ackerveken, 2013).

A sucrose synthase is in the top 50 downregulated genes, one of two suppressed by BR treatment. These are almost identical to Arabidopsis *SUS4*, which contributes to increased sucrose levels when silenced (Bieniawska *et al*., 2007). The downregulation of these genes in potato correlates with the increased sucrose content of BR treated cucumber leaves (Yu *et al*., 2004). Sucrose is known to promote auxin activity (Stokes *et al*., 2013) with auxin and brassinosteroid behaving synergistically in the promotion

of plant growth and development (Vert *et al*., 2008). Auxin is discussed more in **Section 4.6.10**.

Also of particular interest in the down-regulated late BR-response genes is *ERG-1*, down-regulated by BR treatment, with an expression level 0.14 that of mock treated plants (7-fold down). This gene has been previously shown to be rapidly induced by both compatible and incompatible races of *P. infestans*, as well as the bacterium *Erwinia carotovora* ssp. a*trospeptica*, *E. carotovora* culture filtrate, ethylene, and salicylic acid (Dellagi *et al*., 2000). *ERG-1* is assigned to the Mapman functional category signalling (sugar and nutrient physiology), and has similarity to *EXL1* (*EXORDIUM LIKE 1*) and *EXL2* in Arabidopsis. Schroder *et al*. (2012) describe the characterisation of *EXL1* and *2*, with transcripts shown to increase during the night, and during periods of carbon starvation and hypoxia. *EXL1* is shown to suppress BR-mediated growth under low carbon conditions, and the authors hypothesise that these genes are involved in 'shutting down' growth thus aiding the allocation of carbon into essential processes. This would be relevant during infection, when the plant must prioritise the allocation of resources into defence. The function of *ERG-1* in potato is yet to be determined, but it may prove to overlap with that of *EXL1* in Arabidopsis.

4.6 Functional Enrichment in BR-regulated genes

Over-representation analysis (ORA) of the significant gene lists was carried out to reveal trends in functional categories that were differentially regulated by BR treatment, using Mapman software (see **Chapter 2**). Gene function is separated into 35 major categories or 'bins', each with a hierarchy of minor bins within it. Because there are more annotated genes in some categories than others, a random selection of genes is likely to have more representatives for the larger categories purely by chance.

For this reason, it is less informative to look simply at how many genes represent each category in a particular dataset, but instead to look at which categories are enriched relative to their proportion in the reference genome itself. Over-representation analysis of gene function within the BR-treated potato microarray data can be seen in **Figure 4.5**. These functional categories will be discussed in turn, and related to what is already known about brassinosteroid signalling and effects in the literature.

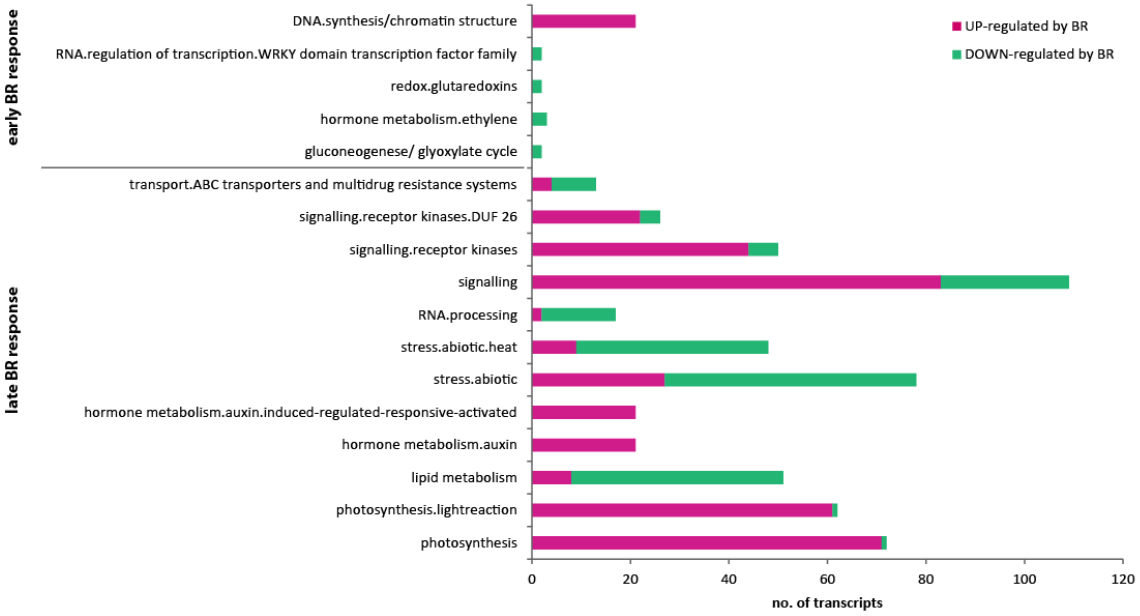

Figure 4.5 Over-Representation Analysis of BR effects on gene functional groups
Over-representation analysis was carried out in Mapman to reveal enrichment of particular gene functions in those genes significantly altered by BR treatment. The colour of the bar indicates the gene function to be over-represented in the up-regulated (magenta) or down-regulated (green) gene set. Calculated using Fishers exact test with Benjamini Hochberg correction of p-values ($p<0.05$).

4.6.1 DNA category

At 3 hours post-EBL treatment there is an enrichment of upregulated genes associated with DNA synthesis/chromatin structure, shown in **Table 4.6**. Over 60% of these are histones; highly conserved proteins in eukaryotes that associate tightly with DNA, and are essential for its packaging into chromosomes. The expression of histone genes is cell-cycle regulated and linked to DNA replication (Rattray and Muller, 2012). Growth is

well characterised as a brassinosteroid response, thus cell division and the associated DNA synthesis would be expected to increase. Histone transcripts have been shown to increase in tobacco cell lines with brassinosteroid treatment (Miyazawa *et al*., 2003) correlating with increased cell proliferation rate. Mussig *et al*. (2002) also show an increase in histone transcripts in wild-type Arabidopsis plants compared to BR-deficient mutants. They observe no direct effect of BR application on histone expression, but the lower hormone concentration used, and the single 1 hour timepoint studied may account for the difference.

The other major gene family up-regulated by BR within the DNA synthesis/chromatin functional category are those coding for mini-chromosome maintenance proteins, which are part of the pre-replicative complex essential for DNA replication (Freeman *et al*., 1999). Up-regulation peaks at 3 hours between 2 and 11-fold and trends towards basal level by 24hours. Any up-regulation still observed at 24 hours did not achieve significance in the microarray data analysis.

*

Table 4.6 Transcripts associated with DNA synthesis/chromatin structure differentially regulated by BR treatment.
Asterisk indicates significant timepoint.

Transcript ID	Uniref based putative functional annotation	fold-change with BR	
		3h*	24h
PGSC0003DMT400016160	Histone H3.2	2.04	1.61
PGSC0003DMT400008733	Origin-recognition complex subunit 6	2.38	1.96
PGSC0003DMT400009751	Histone H2A	2.47	1.66
PGSC0003DMT400071314	Histone H4	2.56	1.68
PGSC0003DMT400005594	Minichromosome maintenance factor	2.69	1.69
PGSC0003DMT400005593	MCM protein	3.03	1.56
PGSC0003DMT400063677	Histone H2A.1	3.07	1.43
PGSC0003DMT400022189	Histone H3.2	3.2	2.07
PGSC0003DMT400021420	DNA replication licensing factor MCM3	3.29	3.19
PGSC0003DMT400028257	Conserved gene of unknown function	3.75	1.69
PGSC0003DMT400035465	Mini-chromosome maintenance protein MCM6	3.92	1.93
PGSC0003DMT400058778	Histone H3.2	3.93	0.93
PGSC0003DMT400035464	Mini-chromosome maintenance protein MCM6	4.23	2.19
PGSC0003DMT400065027	Histone H3.2	4.61	1.12
PGSC0003DMT400063329	70 kDa subunit of replication protein A	6.08	2.48
PGSC0003DMT400064995	Histone H3.2	7.04	3.37
PGSC0003DMT400016201	Histone H3.2	7.21	2.83
PGSC0003DMT400060472	Histone H4	8.34	5.71
PGSC0003DMT400060471	Histone H4	9.81	3.41
PGSC0003DMT400002870	Histone H3.2	10.07	4.44
PGSC0003DMT400064996	Histone H3.2	11.03	3.93

4.6.2 RNA (WRKY transcription factors)

Also over-represented at the 3 hour timepoint is the down-regulation of WRKY genes, one of the largest families of plant-specific transcription factors, perhaps best known for their role in the plant immune response. These are collectively described as a 'Jack of many trades' (Bakshi, 2014) involved in numerous physiological processes such as abiotic stress responses, nutrition and development as well as disease resistance. One of two down-regulated by BR treatment at 3 hours is PGSC0003DMT400000215 (referred to as WRKY23 in the potato genome), a reciprocal best BLAST hit (RBBH) of WRKY22 in Arabidopsis. This transcription factor has been shown to be upregulated during submergence, leading to increased resistance to *P. syringae* in Arabidopsis (Hsu *et al*., 2013). The other WRKY transcription factor significantly down-regulated at 3 hours post-EBL is PGSC0003DMT400072958, a RBBH of AtWRKY57. This is shown to be a negative regulator of JA-induced leaf senescence by Jiang *et al*. (2013). BRs are suggested to promote senescence (He *et al.*, 2001), so the down-regulation of a negative regulator by BR treatment is expected. Both of these WRKY transcription factors are down-regulated approximately 2-fold with BR at 3 hours, and trend towards basal level by 24 hours (see **Table 4.7**).

Table 4.7 RNA (WRKY) transcripts down-regulated by BR at 3 hours
Asterisk indicates significant timepoint

Transcript ID	Uniref based putative functional annotation	fold-change with BR	
		3h*	24h
PGSC0003DMT400072958	Transcription factor	0.42	0.89
PGSC0003DMT400000215	WRKY transcription factor 23	0.48	0.91

4.6.3 Redox genes

The redox category contains two glutaredoxins transcripts down-regulated by BR at 3 hours; both splice variants of the same gene (see **Table 4.8**). Glutaredoxins are a poorly understood family of proteins in both plants and animals, involved in electron transfer. This gene show the highest sequence similarity to *THRUMIN1* in Arabidopsis, a light-regulated gene that codes for an actin accessory protein required for chloroplast motility (Whippo *et al*., 2011).

BRs have been shown to reconfigure the actin cytoskeleton in a similar manner to auxin (Lanza *et al*., 2012). These authors also describe phenotypic overlap between the *actin2* mutant *act2-5* and BZR1 over-expressing or EBL treated Arabidopsis plants, with bending and twisting of above-ground organs and wavy roots, and show that BR signalling is constitutively active in *act2-5* mutant plants. These transcripts remain down-regulated 24 hours after EBL treatment, and are part of the small set of transcripts which are significantly changed at both timepoints.

Table 4.8 Redox (glutaredoxin) transcripts downregulated 3h post-BR
Asterisk indicates timepoints of statistical significance

Transcript ID	Uniref based putative functional annotation	fold-change with BR	
		3h*	24h*
PGSC0003DMT400031969	Electron transporter	0.47	0.48
PGSC0003DMT400031968	Electron transporter	0.47	0.48

4.6.4 Hormone metabolism (ethylene)

The next functional category to be significantly enriched in its down-regulation is hormone metabolism, specifically ethylene. The three transcripts in this category are down-regulated between 2 and 3-fold by BR at 3h, see **Table 4.9**. These are an oxidoreductase, and two putative oxidoreductases, with sequence similarity to the E8 gene in tomato – shown to have a negative effect on ethylene synthesis (Kneissl and Deikman, 1996). Whilst two of these are annotated as 'conserved gene of unknown function', their similarity to the Arabidopsis oxidoreductase At1G49390 leads them to be assigned to the ethylene synthesis/degradation category in MapMan. Brassinosteroid has been shown to increase the synthesis of ethylene in maize (Lim *et al.*, 2002) Arabidopsis (Hansen *et al*., 2009) and cotton (Shi *et al.*, 2006), which may explain the downregulation of a potential negative regulator in potato. These transcripts show the opposite trend at 24 hours post-EBL treatment, with two up-regulated, but these changes were not statistically significant.

Table 4.9 Hormone metabolism (ethylene) transcripts downregulated at 3h post-BR
Asterisk indicates significant timepoint.

Transcript ID	Uniref based putative functional annotation	fold-change with BR	
		3h*	24h
PGSC0003DMT400070453	2-oxoglutarate-dependent dioxygenase	0.34	0.99
PGSC0003DMT400073345	Conserved gene of unknown function	0.42	2.14
PGSC0003DMT400073342	Conserved gene of unknown function	0.44	3.25

4.6.5 Gluconeogenesis genes

Gluconeogenesis is also enriched in the genes down-regulated by BR, see **Table 4.10**. The two transcripts, down-regulated by approximately 2-fold, are citrate synthases – key enzymes involved in the citric acid or Krebs cycle, used to generate energy in cells.

The down-regulation of these genes by BR treatment is perhaps unexpected; the increased growth and development stimulated by brassinosteroids would be expected to demand more energy. In accordance with this, Schroder *et al*. (2014) show reduced TCA cycle activity in Arabidopsis treated with the BR biosynthetic inhibitor BRZ. While this functional category is not enriched in the 24 hour dataset, it is worth noting that two additional gluconeogenesis transcripts significant at 24 hours are both isocitrate lyases, enzymes also involved in the TCA cycle, and these are upregulated 5 and 7-fold with BR treatment. Therefore it is difficult to speculate on TCA cycle activity in BR-treated potato based on this microarray data; it would require analysis of the intermediates to resolve.

Table 4.10 Gluconeogenesis transcripts downregulated by BR at 3h
Asterisk indicates significant timepoint

Transcript ID	Uniref based putative functional annotation	fold-change with BR	
		3h*	24h
PGSC0003DMT400044692	Citrate synthase	0.46	0.89
PGSC0003DMT400044690	Citrate synthase	0.47	1

4.6.6 Transport genes

At 24 hours post-EBL treatment, there is enrichment in the down-regulation of the Mapman bin 'transport.ABC transporters and multidrug resistance systems' (see **Table 4.11**).This gene set consists of 21 transcripts down-regulated by between 2 and 6-fold. The ABC protein superfamily is the largest protein family known in eukaryotes, and play a role in the transport of substances across membranes (Sanchez-Fernandez *et al*., 2001). A review by Martinoia (2002) links them to hormone signalling, light signalling

and ion fluxes, as well as their more classic role as detoxifiers. Of those down-regulated by BR in this study, one is almost identical to *AtMRP5*, an ABC transporter linked to auxin. Knock-out of *MRP5* led to reduced root growth and the accumulation of auxin (Gaedeke *et al*., 2001). Another down-regulated by BR is zinc-induced facilitator 1, again leading to auxin accumulation when silenced (Remy, 2013). ABC transporters have also been linked to plant-microbe interactions and specifically non-host resistance, with expression shown to be induced by salicylic acid and PAMPs (Kang *et al*., 2011), and additionally to stress in the form of herbicide application. Down-regulation of these ABC transporters by BR may have a role in the increased auxin response, and in suppression of PTI. Conversely, Zhou *et al*. (2015) show a subset of ABC transporters to be up-regulated by BR in a variety of crop plants; increasing their capacity to metabolise pesticides thus decreasing residues in the end product.

Table 4.11 ABC transporters differentially regulated by BR treatment at 24h
Asterisk indicates significant timepoint.

Transcript ID	UniRef based putative functional annotation	fold-change with BR	
		3h	24h*
PGSC0003DMT400057099	Multidrug resistance protein ABC transporter family	1	0.18
PGSC0003DMT400060395	Pleiotropic drug resistance protein 2	0.99	0.22
PGSC0003DMT400008521	Protein ZINC INDUCED FACILITATOR-LIKE 1	1.06	0.23
PGSC0003DMT400060396	Pleiotropic drug resistance protein 2	0.65	0.28
PGSC0003DMT400018820	Multidrug/pheromone exporter, MDR family	1.36	0.29
PGSC0003DMT400016069	Multidrug resistance-associated protein 2, 6 (Mrp2, 6)	0.59	0.29
PGSC0003DMT400063063	Multidrug resistance protein	1.34	0.32
PGSC0003DMT400022110	Multidrug resistance protein ABC transporter family	0.76	0.32
PGSC0003DMT400022109	Multidrug resistance protein ABC transporter family	0.89	0.36
PGSC0003DMT400011214	Multidrug resistance protein ABC transporter family	1.34	0.4
PGSC0003DMT400007215	ABC transporter family protein	0.59	0.4
PGSC0003DMT400019317	White-brown-complex ABC transporter family	0.65	0.41
PGSC0003DMT400019318	White-brown-complex ABC transporter family	0.62	0.43
PGSC0003DMT400019316	White-brown-complex ABC transporter family	0.66	0.44
PGSC0003DMT400018812	Multidrug/pheromone exporter, MDR family	1.01	0.44
PGSC0003DMT400004971	White-brown-complex ABC transporter family	1.63	0.45
PGSC0003DMT400045176	P-glycoprotein	0.8	0.46
PGSC0003DMT400045180	P-glycoprotein	0.8	0.46
PGSC0003DMT400004973	White-brown-complex ABC transporter family	1.28	0.47
PGSC0003DMT400047545	White-brown-complex ABC transporter family	1.73	2.58
PGSC0003DMT400047547	White-brown-complex ABC transporter family	1.34	2.6

4.6.7 Signalling genes

The most highly over-represented category at 24 hours is that of 'signalling' associated genes; predominantly up-regulated by BR (83 up compared to 26 down). Within this, receptor kinases (44 up, 6 down), and more specifically domain of unknown function (DUF26) receptor kinases (22 up, 4 down) are particularly enriched.

Among these up-regulated signalling genes are several involved in light signalling; such as NPH3 family proteins, implicated in the integration of light-signalling and auxin flux (Wan *et al*., 2012), ELF4, shown to be involved in maintaining circadian rhythm (Doyle et al, 2002), and LOV domain proteins that serve as light-activated molecular switches (Crosson and Moffat, 2002). Calcium signalling, sugar and nutrient signalling, and G-proteins also have a strong presence. Within the receptor kinases, a large proportion are categorised as having leucine rich repeats – motifs which are involved in protein recognition (Kobe and Kajava, 2001) and have been linked to diverse functions in plants such as disease resistance, hormone perception, and aspects of plant development such as organ size and leaf abscission (Shiu and Bleeker, 2001). Several of the LRR-RKs up-regulated by BR are associated with disease resistance, such as Cf-2.2 and its homologues Hcr2-OA and -OB, which confer resistance to *Cladosporum fulvum* in tomato (Dixon *et al*., 1998), as well as PBS1, required for the recognition of *P. syringae* effector AvrPphB in Arabidopsis (Swidderski and Innes, 2001). This may go some way in explaining the contradictory effects that BR treatment has on disease resistance. Despite the antagonism now known to occur between BR signalling and PTI, Nakashita *et al*. (2003) show brassinosteroid treatment to enhance disease resistance in tobacco and rice. It may be that while aspects of PTI are compromised, the upregulation of some LRR-RKs involved in effector recognition means that ETI is enhanced to a degree. This could in theory be an evolutionary response in plants to pathogen manipulation of the BR pathway: if effectors activate the pathway to suppress immunity, having resistance genes under the control of the BR pathway would counteract this. SdR2, the resistance gene responsible for recognition of AVR2, has a putative BZR1 binding site in its promotor region (Shaista Naqvi, personal

communication) which could indicate positive regulation by BR. Additionally, a transcript with similarity to the resistance gene R2 is upregulated by BR in the microarray data. PGSC0003DMT400030047 has 80% amino acid identity to R2-like, 79% to R2 itself, and is 2-fold up-regulated by BR after 24 hours. *Solanum tuberosum* cv. Desiree plants do not recognise AVR2, so this transcript may potentially recognise a variant of the effector, another effector entirely, or may not code for a functional resistance protein in this species. However, it would be interesting to look at R2 expression in its native plant *Solanum demissum*, and determine whether BR treatment has any effect on expression or AVR2 recognition. Signalling transcripts regulated by BR treatment in this study can be found in **Table 4.12.**

Table 4.12 Signalling transcripts differentially regulated by BR treatment at 24h
Asterisk indicates significant timepoint.

Transcript ID	Uniref based putative functional annotation	fold-change with BR	
		3h	24h*
PGSC0003DMT400067920	UPA22	1.11	15.08
PGSC0003DMT400068851	PAS/LOV protein A	0.57	6.35
PGSC0003DMT400068848	Twin lov protein	0.56	6.11
PGSC0003DMT400094671	Leucine-rich repeat family protein	2.48	5.24
PGSC0003DMT400043544	Serine-threonine protein kinase, plant-type	1.55	4.33
PGSC0003DMT400049615	S-locus-specific glycoprotein S13	1.14	4.28
PGSC0003DMT400068849	Twin lov protein	0.69	4.25
PGSC0003DMT400072046	Conserved gene of unknown function	3.29	3.87
PGSC0003DMT400036789	ATP binding protein	1.89	3.8
PGSC0003DMT400036084	RabGAP/TBC domain-containing protein	0.78	3.64
PGSC0003DMT400048899	Photoreceptor-interacting protein	0.95	3.25
PGSC0003DMT400043020	Receptor protein kinase CLAVATA1	1.69	3.25
PGSC0003DMT400070026	ATP binding / carbohydrate binding / kinase…	1.32	3.2
PGSC0003DMT400065542	Glutamate receptor 3 plant	1.2	3.17
PGSC0003DMT400065543	Glutamate receptor 3 plant	1.02	3.11
PGSC0003DMT400050471	SF16	1.44	3.09
PGSC0003DMT400053693	Conserved gene of unknown function	1.58	3.04
PGSC0003DMT400073675	Conserved gene of unknown function	1.42	3.03
PGSC0003DMT400037043	Calmodulin binding protein	0.93	2.94
PGSC0003DMT400073677	Conserved gene of unknown function	1.44	2.94
PGSC0003DMT400073678	Conserved gene of unknown function	1.45	2.93

PGSC0003DMT400012679	S-locus-specific glycoprotein S13	1	2.88
PGSC0003DMT400012677	S-locus-specific glycoprotein S6	1.15	2.87
PGSC0003DMT400068850	Twin lov protein	0.40*	2.81
PGSC0003DMT400068207	Avr9/Cf-9 rapidly elicited protein 141	1.67	2.74
PGSC0003DMT400012062	Hcr2-0A	1.6	2.74
PGSC0003DMT400008336	Leucine Rich Repeat family protein	1.44	2.74
PGSC0003DMT400033250	Calmodulin	1.28	2.74
PGSC0003DMT400024269	Receptor protein kinase CLAVATA1	1.74	2.73
PGSC0003DMT400066268	Kinase	1.75	2.66
PGSC0003DMT400033249	Calmodulin	1.62	2.65
PGSC0003DMT400014122	Hcr2-0A	2.16	2.65
PGSC0003DMT400031088	Pirin	0.5	2.64
PGSC0003DMT400065541	Glutamate receptor 3 plant	1.05	2.63
PGSC0003DMT400021179	Calmodulin binding protein	2.16	2.6
PGSC0003DMT400076879	CCHC-type integrase	1.13	2.59
PGSC0003DMT400012061	Hcr2-0A	1.55	2.58
PGSC0003DMT400036504	Receptor kinase	1.45	2.58
PGSC0003DMT400061303	Hcr2-0B	1.49	2.55
PGSC0003DMT400049201	Kinase family protein	0.99	2.53
PGSC0003DMT400036051	S-locus-specific glycoprotein S6	0.86	2.53
PGSC0003DMT400016935	EARLY flowering 4 protein	0.96	2.51
PGSC0003DMT400062337	Receptor protein kinase zmpk1	2.01	2.51
PGSC0003DMT400014127	Cf-2.2	2.01	2.5
PGSC0003DMT400049617	Conserved gene of unknown function	0.98	2.45
PGSC0003DMT400062333	Receptor protein kinase zmpk1	2.01	2.44
PGSC0003DMT400047608	GTP-binding protein alpha subunit, gna	0.8	2.44
PGSC0003DMT400044505	Receptor-like kinase	1.39	2.39
PGSC0003DMT400089865	EARLY flowering 4 protein	0.96	2.39
PGSC0003DMT400010940	Disease resistance protein	0.89	2.38
PGSC0003DMT400046279	Hcr2-p3	1.46	2.37
PGSC0003DMT400044503	Receptor-like kinase	1.41	2.36
PGSC0003DMT400029528	Phototropic-responsive NPH3 family protein	1.45	2.36
PGSC0003DMT400044506	Receptor-like kinase	1.58	2.34
PGSC0003DMT400045690	Conserved gene of unknown function	1.68	2.32
PGSC0003DMT400044735	Receptor-like kinase	1.35	2.32
PGSC0003DMT400003225	Receptor kinase	1.02	2.3
PGSC0003DMT400066620	Calmodulin-binding protein	0.78	2.29
PGSC0003DMT400007482	Serine/threonine-protein kinase bri1	0.58	2.28
PGSC0003DMT400022302	ROP	1.29	2.27
PGSC0003DMT400044608	Receptor-like kinase	1.45	2.27
PGSC0003DMT400032060	ATP binding protein	1.32	2.25
PGSC0003DMT400045691	Receptor protein kinase	1.66	2.24
PGSC0003DMT400047030	Serine/threonine-protein kinase PBS1	0.94	2.23
PGSC0003DMT400062335	Receptor protein kinase zmpk1	1.75	2.22
PGSC0003DMT400069760	Phosphoinositide-specific phospholipase C	1.04	2.19
PGSC0003DMT400080167	RAB7A	0.81	2.17
PGSC0003DMT400051789	RPM1 interacting protein 4 transcript 2	1.14	2.17

PGSC0003DMT400047609	GTP-binding protein alpha subunit, gna	0.84	2.16
PGSC0003DMT400021178	Calmodulin binding protein	1.98	2.15
PGSC0003DMT400078562	Receptor kinase	1.92	2.14
PGSC0003DMT400045475	PAS/LOV protein A	0.72	2.1
PGSC0003DMT400047610	GTP-binding protein alpha subunit, gna	0.99	2.09
PGSC0003DMT400011870	Rapid alkalinization factor 1	1.15	2.08
PGSC0003DMT400065536	Glutamate receptor 3 plant	1.12	2.08
PGSC0003DMT400090017	Serine-threonine protein kinase, plant-type	0.96	2.08
PGSC0003DMT400068927	Apple; Protein kinase; EGF-like, subtype 2	0.95	2.08
PGSC0003DMT400041342	Calmodulin	1.14	2.07
PGSC0003DMT400045476	Twin lov protein	0.7	2.05
PGSC0003DMT400045473	Twin lov protein	0.77	2.03
PGSC0003DMT400065535	Glutamate receptor 3 plant	1.12	2.02
PGSC0003DMT400021325	NAD dependent epimerase/dehydratase	1.08	2.01
PGSC0003DMT400017800	Protein kinase family protein	0.84	0.49
PGSC0003DMT400048609	Ccd1	0.85	0.47
PGSC0003DMT400043037	Ubiquitin-protein ligase	1.6	0.47
PGSC0003DMT400073081	Wd40 protein	0.88	0.46
PGSC0003DMT400001492	Calcium ion binding protein	0.63	0.46
PGSC0003DMT400057823	Serine/threonine-protein kinase bri1	0.94	0.45
PGSC0003DMT400001493	Calcium ion binding protein	0.54	0.44
PGSC0003DMT400038052	Cytohesin 1, 2, 3	0.84	0.41
PGSC0003DMT400015454	Ubiquitin ligase protein cop1	0.21	0.4
PGSC0003DMT400017803	Cysteine-rich receptor kinase 43	0.95	0.39
PGSC0003DMT400057716	WD-repeat protein	0.83	0.39
PGSC0003DMT400004617	Conserved gene of unknown function	0.88	0.37
PGSC0003DMT400089961	Conserved gene of unknown function	0.83	0.37
PGSC0003DMT400004142	39 kDa EF-Hand containing protein	1.02	0.34
PGSC0003DMT400044455	Conserved gene of unknown function	1.62	0.31
PGSC0003DMT400004141	39 kDa EF-Hand containing protein	0.71	0.3
PGSC0003DMT400016945	Phosphoinositide-specific phospholipase C	1.19	0.28
PGSC0003DMT400079159	Conserved gene of unknown function	1.36	0.25
PGSC0003DMT400074356	ATP binding protein	0.45	0.2
PGSC0003DMT400055528	Calcium lipid binding protein	1.03	0.19
PGSC0003DMT400074357	ATP binding protein	0.66	0.18
PGSC0003DMT400016946	Phosphoinositide-specific phospholipase C	1.15	0.17
PGSC0003DMT400032756	Erg-1	0.75	0.14
PGSC0003DMT400032859	NOI	0.83	0.13

4.6.8 RNA processing genes

Genes associated with RNA processing are over-represented in the down-regulated transcripts at 24 hours post-BR treatment. This gene list consists of several splicing

factors and spliceosome subunits, as well as DICER-1, a highly conserved enzyme in eukaryotes responsible for generating small regulatory RNAs, or microRNAs. These are involved in post-translational gene regulation, and have a key role in negotiating developmental transitions (Margis *et al*., 2006), such as organ differentiation and flowering time. Changes in microRNA levels, and differences in alternative splicing, have been linked to hormone signalling, and response to biotic and abiotic stress (Kruszka *et al*., 2012). RNA processing transcripts significantly affected in this study can be seen in **Table 4.13**.

Table 4.13 RNA processing transcripts differentially regulated by BR at 24h
Asterisk indicates significant timepoint.

Transcript ID	Uniref based putative functional annotation	fold-change with BR	
		3h	24h*
PGSC0003DMT400068226	U2 snrnp auxiliary factor, small subunit	1.12	0.19
PGSC0003DMT400074732	Poly(A) polymerase	1.15	0.3
PGSC0003DMT400063703	Arginine/serine-rich splicing factor	0.73	0.34
PGSC0003DMT400074733	Poly(A) polymerase	1.35	0.35
PGSC0003DMT400029924	Endoribonuclease	1.65	0.36
PGSC0003DMT400040069	Splicing factor U2af large subunit B	1.56	0.38
PGSC0003DMT400001810	Dicer-1	1	0.4
PGSC0003DMT400001808	Ribonuclease 3 3	1.07	0.44
PGSC0003DMT400054659	RNA 3' terminal phosphate cyclase	1.55	0.44
PGSC0003DMT400079553	RNA-binding region RNP-1 & Splicing factor PWI	1.58	0.46
PGSC0003DMT400010895	DEAD-box ATP-dependent RNA helicase 48	0.82	0.46
PGSC0003DMT400001809	Ribonuclease 3 3	1.26	0.48
PGSC0003DMT400079552	RNA-binding region RNP-1 & Splicing factor PWI	1.67	0.49
PGSC0003DMT400069041	Splicing factor 3B subunit	1.08	0.49
PGSC0003DMT400063701	Arginine/serine-rich splicing factor	0.85	0.49
PGSC0003DMT400002898	RNA binding	0.88	2.49
PGSC0003DMT400015091	CCR4-associated factor	0.99	2.61

4.6.9 Abiotic stress genes

89 transcript associated with abiotic stress are differentially regulated in the late response to BR, with a slight majority (51) showing down-regulation (see **Table 4.14**). Most of these are assigned to heat stress, and are heat shock proteins. Heat shock proteins are molecular chaperones which maintain normal protein folding and assembly under stress conditions (Ye *et al*., 2012), and are implicated in tolerance to a variety of abiotic stresses such as salt, drought and cold as well as the heat shock for which they are named. Abscisic acid (ABA) is the primary phytohormone responsible for abiotic stress signalling and adaptation in plants (Tuteja, 2007). Antagonistic crosstalk between BR and ABA signalling is well-documented in the literature (Chung *et al*., 2014), with ABA inhibiting BR signalling outputs (Zhang *et al*. 2009), and BR inhibiting ABA responses (Ryu *et al*. 2014). This could provide an explanation for the decrease in expression levels of abiotic stress genes seen with BR treatment in this microarray.

BR insensitive plants, *bri1* mutants, have been shown to have constitutively higher expression of stress-inducible genes (Kim *et al*., 2010) and are more tolerant to cold. Conversely, BRI1 over-expressing plants were more sensitive to stress, with lower expression of stress-inducible genes. Thus, BR treatment would be expected to mimic the over-expression of BRI1, leading to the down-regulation of stress genes.

Despite this, several authors have shown an increase in stress tolerance with BR treatment, which seems contradictory. Dhaubadel *et al*. (1999) show that treatment with BR enhances the thermotolerance of both tomato and *Brassica napus*, with increased levels of heat shock proteins which is in striking contrast to results in this study. Kagale *et al*. (2007) add to this and show increased tolerance to cold and salt

stress with BR. Interestingly, they also describe that BR-deficient mutants such as *dwf4* and *det2-1* still accumulate heat shock proteins as normal under heat stress. This suggests that although BRs have impact on HSP levels, they are not the main point of control. Relative levels of BR may be key in this balance; Kim *et al*. (2010) note that only a very low concentration of BR (<1nM) was able to increase tolerance to cold in Arabidopsis, with no benefit of higher concentrations

In addition to down-regulated stress genes, BR also increased the expression of several stress-related transcripts in this study. Two of these are *PYL1* and *PYL4*, receptors for abscisic acid, which would be expected to result in stronger stress responses and increased tolerance.

The three most strongly up-regulated genes in this category are 'major latex' genes. The function of these has not been determined, but they have been positively linked to both cell expansion in developing peach and cucumber (Ruperti *et al.*, 2002), as well as response to salt and fungal elicitors (Chen *et al*., 2010). Also up-regulated is ERD15; a gene identified from a wild tomato species *Solanum pennelli* which is drought and salt tolerant (Ziaf *et al*., 2011). The authors show this to enhance stress tolerance in tobacco plants when overexpressed. Interestingly ERD15 has been shown to have the opposite effect in Arabidopsis. Overexpression led to decreased sensitivity to ABA, therefore reduced tolerance to drought (Kariola, 2006).

It is evident that the effects of BR on abiotic stress responses are not clear cut; there may be sensitivity to gradients, differential effects in different tissues, and input from other signalling pathways which will contribute to the outcome. Studying the tolerance of *S. tuberosum* to stresses such as heat, cold and drought, in combination with BR

treatment at various concentrations would be an informative future study. The apparent contradiction seen in the gene lists in this study, with some stress response genes upregulated and others down-regulated by BR, makes it difficult to speculate on what the final effect on stress tolerance might be in potato.

Table 4.14 Abiotic stress transcripts differentially regulated by BR at 24h
Asterisk indicates significant timepoint

		fold-change with BR	
Transcript ID	**Uniref based putative functional annotation**	**3h**	**24h***
PGSC0003DMT400021142	Class II small heat shock protein Le-HSP17.6	0.66	0.05
PGSC0003DMT400037083	Low-temperature-induced 65 kDa protein	0.52	0.09
PGSC0003DMT400078006	17.6 kD class I small heat shock protein	0.43	0.1
PGSC0003DMT400014216	Heat shock protein	0.36	0.12
PGSC0003DMT400001180	Heat shock cognate 70 kDa protein	0.53	0.13
PGSC0003DMT400001182	Heat shock cognate 70 kDa protein	0.57	0.13
PGSC0003DMT400030387	Chloroplast small heat shock protein class I	0.45	0.13
PGSC0003DMT400001181	Heat shock cognate 70 kDa protein	0.56	0.14
PGSC0003DMT400030382	Chloroplast small heat shock protein class I	0.46	0.14
PGSC0003DMT400074375	Heat shock protein 90	0.59	0.14
PGSC0003DMT400007728	DNAJ protein	0.53	0.15
PGSC0003DMT400012249	Mitochondrial small heat shock protein	0.64	0.15
PGSC0003DMT400063352	101 kDa heat shock protein	0.58	0.16
PGSC0003DMT400030385	Chloroplast small heat shock protein class I	0.54	0.17
PGSC0003DMT400053402	Heat-shock protein	1.4	0.18
PGSC0003DMT400084231	Universal stress protein family protein	0.3	0.19
PGSC0003DMT400024594	Heat shock protein 83	0.92	0.19
PGSC0003DMT400078202	Hsp20.1 protein	0.65	0.19
PGSC0003DMT400071607	J-domain protein	0.94	0.2
PGSC0003DMT400014217	Heat shock protein 83	1.04	0.21
PGSC0003DMT400077357	Heat shock protein 70kD	0.8	0.22
PGSC0003DMT400078201	17.6 kD class I small heat shock protein	0.61	0.23
PGSC0003DMT400078007	17.6 kD class I small heat shock protein	1.11	0.24
PGSC0003DMT400055930	Furin	0.79	0.25
PGSC0003DMT400074374	Heat shock protein 83	0.82	0.26
PGSC0003DMT400071337	Hsc70	0.52	0.27
PGSC0003DMT400042378	Conserved gene of unknown function	1.09	0.28
PGSC0003DMT400078163	Heat shock cognate 70 kDa protein 1	0.68	0.29
PGSC0003DMT400031252	Small heat shock protein	1.18	0.3
PGSC0003DMT400064990	DnaJ	1.31	0.33
PGSC0003DMT400031253	Small heat shock protein	0.78	0.34
PGSC0003DMT400024267	DnaJ	1.08	0.36
PGSC0003DMT400036855	Heat shock protein	0.8	0.36

PGSC0003DMT400044066	Hsp20.1 protein	0.91	0.37
PGSC0003DMT400008101	Heat shock 70 kDa protein, mitochondrial	1.25	0.4
PGSC0003DMT400065838	Fiber protein Fb2	1.29	0.4
PGSC0003DMT400058588	Conserved gene of unknown function	0.86	0.41
PGSC0003DMT400016040	Heat shock protein binding protein	1.17	0.41
PGSC0003DMT400008100	Heat shock 70 kDa protein, mitochondrial	1.12	0.43
PGSC0003DMT400007975	COR414-TM1	0.88	0.43
PGSC0003DMT400044353	S-adenosylmethionine-dependent methyltransferase	0.95	0.44
PGSC0003DMT400007974	COR414-TM1	0.73	0.44
PGSC0003DMT400001927	Response to dessication RD2	1.13	0.45
PGSC0003DMT400081223	Protein SIS1	0.9	0.45
PGSC0003DMT400065839	Fiber protein Fb2	1.8	0.47
PGSC0003DMT400008099	Heat shock 70 kDa protein, mitochondrial	0.93	0.47
PGSC0003DMT400008098	Heat shock 70 kDa protein, mitochondrial	0.94	0.47
PGSC0003DMT400027701	Heat shock 70 kDa protein, mitochondrial	0.7	0.48
PGSC0003DMT400027703	Heat shock 70 kDa protein, mitochondrial	0.64	0.48
PGSC0003DMT400066224	Cell division cycle protein	1.23	0.48
PGSC0003DMT400027704	Heat shock 70 kDa protein, mitochondrial	0.7	0.48
PGSC0003DMT400068739	Conserved gene of unknown function	0.78	2
PGSC0003DMT400019732	ERD15	0.98	2.05
PGSC0003DMT400012888	Pathogenesis-induced protein	1.42	2.1
PGSC0003DMT400023627	Chaperone protein dnaJ	1.46	2.29
PGSC0003DMT400068741	Conserved gene of unknown function	0.72	2.34
PGSC0003DMT400019731	ERD15	1.1	2.4
PGSC0003DMT400009601	Nicotiana tabacum wound inducive mRNA	1.22	2.41
PGSC0003DMT400019730	ERD15	1.09	2.45
PGSC0003DMT400041080	Abscisic acid receptor PYL4	0.68	2.5
PGSC0003DMT400023625	Chaperone protein dnaJ	0.92	2.51
PGSC0003DMT400063773	DnaJ	1.08	2.51
PGSC0003DMT400023624	Chaperone protein dnaJ	0.94	2.55
PGSC0003DMT400077372	Gamma-glutamyl transferase	1.6	3.02
PGSC0003DMT400030605	Universal stress protein family protein	0.95	3.12
PGSC0003DMT400045156	Abscisic acid receptor PYL1	0.96	3.13
PGSC0003DMT400028658	Abscisic acid receptor PYL4	0.98	3.14
PGSC0003DMT400045253	Universal stress protein family protein	1.24	3.45
PGSC0003DMT400042448	15.4 kDa class V heat shock protein	0.89	3.45
PGSC0003DMT400061535	Abscisic acid receptor PYL4	0.91	3.83
PGSC0003DMT400023512	CAPIP1	1.43	4.15
PGSC0003DMT400023511	CAPIP1	1.51	4.32
PGSC0003DMT400063774	DnaJ	0.93	4.36
PGSC0003DMT400044224	Rhicadhesin receptor	1.1	4.81
PGSC0003DMT400036852	DnaJ protein	1.01	6.29
PGSC0003DMT400073915	Major latex	1.43	22.48
PGSC0003DMT400022721	Major latex	1.58	32.01
PGSC0003DMT400031542	Major latex	1.7	34.52

4.6.10 Auxin-associated genes

Auxin-associated genes are significantly enriched in the up-regulated transcripts at 24 hours, with 21 transcripts increased by BR and none down-regulated (see **Table 4.15**). 19 of these are SAURs (Small Auxin-Upregulated RNAs), and are expressed at a level between 2 and 7-fold higher than untreated potato plants. They are a large gene family, consisting of 78 members in Arabidopsis, yet their function is largely unknown, compounded largely by a lack of phenotype in knockout lines and potential redundancy between family members (Kant and Rothstein, 2009). Arabidopsis SAUR76 is linked to cell elongation in roots (Markakis, 2013), and SAUR39 has been shown to negatively regulate auxin synthesis (Kant and Rothstein 2009), akin to the negative feedback shown by BR treatment on BR biosynthesis. The closely related SAURs 19-24 have been shown to positively regulate leaf size, and affect auxin transport (Spartz, 2012).

Auxin and brassinosteroids have synergistic effects on plant physiology (Nemhauser *et al.*, 2004), with a large number of genes shown to be under the control of both hormones, and the requirement of both pathways to be intact for optimal growth response. The two signalling pathways have been shown to be integrated by BIN2, the kinase better known for its role in the brassinosteroid pathway. BIN2 interacts with the auxin response factor ARF2 (Vert *et al*. 2008), one of a family of transcription factors known to activate or repress genes in response to auxin (Guilfoyle *et al*., 2007). BIN2 is proposed to inactivate this particular repressor ARF, leading to increased expression of auxin-response genes. Notably, potato ARF6 is upregulated by BR in this study by 3 fold. ARF6 has been shown to positively regulate growth in Arabidopsis, linked to stem elongation and floral development by Liu *et al*. (2014).

Interestingly, auxin has been shown to have inhibitory effects on the hypersensitive response in tobacco (Chang *et al*., 2015), providing another example of the crosstalk between growth and defence.

Table 4.15 Auxin-associated transcripts upregulated by BR at 24 hours
Asterisk indicates significant timepoint.

Transcript ID	Uniref based putative functional annotation	fold-change with BR	
		3h	24h*
PGSC0003DMT400042686	Auxin-induced SAUR	2.66	7.48
PGSC0003DMT400034661	Auxin-induced SAUR	2.41	7.39
PGSC0003DMT400034480	Auxin-induced SAUR	3.59	6.6
PGSC0003DMT400042747	Auxin-induced SAUR	2.13	6.37
PGSC0003DMT400004089	SAUR family protein	2.45	6.37
PGSC0003DMT400042870	Dopamine beta-monooxygenase	1.65	6.04
PGSC0003DMT400034653	Auxin-induced SAUR	3.56	5.95
PGSC0003DMT400042692	Auxin-induced SAUR	1.95	5.21
PGSC0003DMT400042697	Auxin-induced SAUR	2.62	5.04
PGSC0003DMT400042693	Auxin-induced SAUR	1.8	4.65
PGSC0003DMT400042691	Auxin-induced SAUR	1.89	4.43
PGSC0003DMT400042688	Auxin-induced SAUR	2.71	4.1
PGSC0003DMT400042695	Auxin-induced SAUR	2.71	4.06
PGSC0003DMT400004187	SAUR family protein	2.1	3.88
PGSC0003DMT400004070	Auxin-induced SAUR	1.62	3.62
PGSC0003DMT400012852	Auxin-induced SAUR	1.84	3.41
PGSC0003DMT400091411	Auxin-induced in root cultures protein 12	1.65	2.99
PGSC0003DMT400042690	Auxin-induced SAUR	1.49	2.56
PGSC0003DMT400056541	Auxin-induced SAUR	1.4	2.26
PGSC0003DMT400056538	Auxin-induced SAUR	1.21	2.14
PGSC0003DMT400053349	SAUR family protein	1.22	2.07

4.6.11 Lipid metabolism genes

51 differentially-regulated transcripts with BR treatment are associated with lipid metabolism, with the majority (42) down-regulated. Many of these are linked to lipid transfer, lipid degradation, and metabolism of 'exotics' such as steroids. Pokotylo *et al*. (2014) describe the increased oil content of *Brassica napus* seeds with BR treatment, showing modulation in fatty acid compositition. Again there are links to stress; with

the authors showing that BR treatment restores seed oil content in salt stressed plants to normal levels. This could potentially be the result of reduced lipid metabolism; less degradation and turn-over, and is also in support of the downregulation of stress responses already discussed.

3 of the down-regulated transcripts are flavonol 4'-sulfotransferases, most similar to the brassinosteroid sulfotransferase ST1 in Arabidopsis. ST1, and its orthologue in *Brassica napus* ST3, have been implicated in the inactivation of brassinosteroids (Rouleau *et al*. 1999). The authors show that sulfonation of 24-epibrassinoslide abolishes its biological activity. These and other sulfotransferases have been shown to be upregulated by SA treatment, as well as bacterial challenge and fungal elicitors (Lacomme *et al*. 1996, Masuda *et al*. 1996). This would result in reduced activity of brassinosteroid signalling and could potentially play a role in the plant prioritising resources into defence rather than growth.

The down-regulated gene set in lipid metabolism also features several acyltransferases; enzymes which catalyse the transfer of an acyl group to compounds. Brassinosteroids have been shown to be acylated (Schneider *et al*. 2012), and several studies have shown the overexpression of brassinosteroid acyltransferases to result in the dwarf phenotypes characteristic of BR deficient plants (Schneider *et al*. 2012, Roh *et al*. 2012, Choi *et al*. 2013).

Interestingly, a potato transcript with high similarity to AtKCS1, 3-ketoacyl-CoA synthase 10, is down-regulated at 24 hours in this microarray. This is surprising based on work by Goda *et al*. (2002) who show KCS1 to be up-regulated by BR treatment in Arabidopsis. BR appears to have increased this transcript initially in potato, with

expression 1.6 fold higher with BR at 3h (so deemed not statistically significant), but it is down-regulated to 0.32 that of mock treated plants at 24h. This may be a functionally different gene in potato so responds differently to BR treatment, it may have different kinetics in different species, or it may be a result of the different treatment conditions used, with Goda *et al*. using seedlings treated with only 10nM BL as opposed to the 50µM EBL used in this study. Fold-change information can be seen in **Table 4.16**.

Table 4.16 Lipid metabolism transcripts differentially regulated by BR at 24h
Asterisk indicates significant timepoint.

Transcript ID	Uniref based putative functional annotation	fold-change with BR	
		3h	24h*
PGSC0003DMT400033413	Non-specific lipid-transfer protein	0.27	0.05
PGSC0003DMT400033411	Non-specific lipid-transfer protein	0.22	0.06
PGSC0003DMT400033415	Non-specific lipid-transfer protein	0.28	0.06
PGSC0003DMT400033412	Non-specific lipid-transfer protein	0.2	0.06
PGSC0003DMT400073687	Acyl-protein thioesterase	0.55	0.07
PGSC0003DMT400033409	Non-specific lipid-transfer protein	1.17	0.1
PGSC0003DMT400005893	Triacylglycerol lipase	1.1	0.11
PGSC0003DMT400000153	GPAT	1.18	0.16
PGSC0003DMT400006324	Non-specific lipid-transfer protein	0.56	0.17
PGSC0003DMT400031201	Non-specific lipid-transfer protein 2	1.55	0.17
PGSC0003DMT400000152	GPAT	1.25	0.19
PGSC0003DMT400026502	Triacylglycerol lipase	0.54	0.2
PGSC0003DMT400031202	Non-specific lipid-transfer protein 1	1.72	0.2
PGSC0003DMT400026501	Triacylglycerol lipase	0.56	0.2
PGSC0003DMT400037950	Adenosine monophosphate binding protein 1	1.06	0.25
PGSC0003DMT400063103	Diacylglycerol kinase, theta	0.9	0.26
PGSC0003DMT400028048	Acyltransferase	1.02	0.29
PGSC0003DMT400012973	Sphingosine kinase	0.63	0.29
PGSC0003DMT400072562	Digalactosyldiacylglycerol synthase	1.15	0.3
PGSC0003DMT400031199	Non-specific lipid-transfer protein	0.7	0.3
PGSC0003DMT400033417	Non-specific lipid-transfer protein	0.69	0.3
PGSC0003DMT400063104	Diacylglycerol kinase, theta	0.8	0.31
PGSC0003DMT400066060	Flavonol 4'-sulfotransferase	1.13	0.31
PGSC0003DMT400031200	Non-specific lipid-transfer protein	0.69	0.31

PGSC0003DMT400028047	3-ketoacyl-CoA synthase 10	1.6	0.32
PGSC0003DMT400062607	Anthranilate N-benzoyltransferase protein	0.78	0.33
PGSC0003DMT400026550	Triacylglycerol lipase	0.57	0.33
PGSC0003DMT400068121	Palmitoyl-acyl carrier protein thioesterase	1.53	0.35
PGSC0003DMT400015632	Acyltransferase	1	0.36
PGSC0003DMT400096856	Flavonol 4'-sulfotransferase	0.73	0.36
PGSC0003DMT400015633	Acyltransferase	0.99	0.37
PGSC0003DMT400004106	Phospholipase C	0.72	0.39
PGSC0003DMT400014241	Flavonol 4'-sulfotransferase	0.77	0.39
PGSC0003DMT400072874	Flavonol 4'-sulfotransferase	0.75	0.39
PGSC0003DMT400083974	Hydrolase, alpha/beta fold family protein	1.22	0.4
PGSC0003DMT400012974	Sphingosine kinase	0.74	0.4
PGSC0003DMT400053145	Acyl-coenzyme A oxidase 2, peroxisomal	0.69	0.45
PGSC0003DMT400066828	Conserved gene of unknown function	1.12	0.46
PGSC0003DMT400032992	Gene of unknown function	0.8	0.47
PGSC0003DMT400064400	Benzoquinone reductase	1.29	0.48
PGSC0003DMT400035715	Formiminotransferase-cyclodeaminase	1.57	0.48
PGSC0003DMT400081374	Esterase/lipase/thioesterase family protein	0.61	0.49
PGSC0003DMT400032993	Gene of unknown function	0.88	0.49
PGSC0003DMT400027340	CMV 1a interacting protein 1	1.25	2.14
PGSC0003DMT400069803	Dihydrolipoyl dehydrogenase	0.91	2.56
PGSC0003DMT400063553	Taz protein	1.09	2.68
PGSC0003DMT400080247	Non-specific lipid-transfer protein	0.87	2.75
PGSC0003DMT400063554	Taz protein	1	3.11
PGSC0003DMT400059705	Phospholipase C	0.93	3.14
PGSC0003DMT400061895	AMP dependent ligase	1.66	3.3
PGSC0003DMT400073750	Microsomal omega-6-desaturase	1.53	5.19
PGSC0003DMT400077598	Esterase/lipase/thioesterase family protein	1.51	6.16

4.6.12 Photosynthesis genes

One of the largest over-represented categories differentially regulated by BR is photosynthesis (71 transcripts up-regulated, only 1 down), and within this, 62 are assigned to the sub-category light reactions (see **Table 4.17**). The most highly up-regulated of these are the chlorophyll a-b binding proteins, of which one, *CAB50*, was selected as a marker gene as already described in **Section 4.3**. Xia *et al*. (2009) describe the enhanced photosynthesis seen in cucumber with EBL treatment; with increased carbon assimilation and expression of photosynthesis genes. Wu *et al*. (2008) also describe increased photosynthetic efficiency in transgenic rice plants with overactive

brassinosteroid biosynthesis. This could be a result of the up-regulation of chlorophyll a-b binding proteins, which are required for light harvesting in complex with chlorophyll, and are one of the most abundant membrane proteins in nature (Xu *et al*., 2012).

Table 4.17 Photosynthesis transcripts differentially regulated by BR at 24h
Asterisk indicates significant timepoint

Transcript ID	Uniref based putative functional annotation	fold-change with BR	
		3h	24h*
PGSC0003DMT400043054	Chlorophyll a-b binding protein 50, chloroplastic	1.96	13.37
PGSC0003DMT400034896	Chlorophyll a-b binding protein 3C, chloroplastic	2.11	13.27
PGSC0003DMT400034897	Chlorophyll a-b binding protein 3C, chloroplastic	2.17	12.81
PGSC0003DMT400035007	Chlorophyll a-b binding protein 3C, chloroplastic	2.71	11.22
PGSC0003DMT400034898	Chlorophyll a-b binding protein 3C, chloroplastic	1.18	8.07
PGSC0003DMT400034895	Chlorophyll a-b binding protein 3C, chloroplastic	1.59	7.04
PGSC0003DMT400059995	Chlorophyll a-b binding protein 6A, chloroplastic	2.28	4.71
PGSC0003DMT400031868	Electron carrier	2.6	4.68
PGSC0003DMT400052875	H-Protein	1.84	4.51
PGSC0003DMT400034892	Chlorophyll a-b binding protein 3C, chloroplastic	1.28	4.36
PGSC0003DMT400034893	Chlorophyll a-b binding protein 3C	1.53	4.2
PGSC0003DMT400011382	Light-harvesting complex I protein Lhca5	3.29	4.19
PGSC0003DMT400061950	Fructose-1,6-bisphosphatase, cytosolic	1.61	4.17
PGSC0003DMT400049550	Chlorophyll a-b binding protein 13, chloroplastic	1.55	3.96
PGSC0003DMT400054481	Photosystem I subunit III	2.25	3.89
PGSC0003DMT400050232	Chlorophyll a/b binding protein	2.05	3.84
PGSC0003DMT400052662	Conserved gene of unknown function	1.46	3.78
PGSC0003DMT400073693	Chloroplast post-illumination chlorophyll fluorescence increase protein	1.03	3.46
PGSC0003DMT400034899	Chlorophyll a-b binding protein 3C, chloroplastic	1.61	3.45
PGSC0003DMT400019031	Chlorophyll a/b-binding protein PS II-Type I	1.85	3.39
PGSC0003DMT400054482	Photosystem I subunit III	2.26	3.05
PGSC0003DMT400021388	Chlorophyll a-b binding protein 1B, chloroplastic	1.48	3.02
PGSC0003DMT400062138	Ribulose bisphosphate carboxylase small chain C, chloroplastic	1.97	3.02
PGSC0003DMT400082424	PsbP domain-containing protein 2, chloroplastic	1.33	2.97
PGSC0003DMT400042546	PSI-H	2.34	2.85
PGSC0003DMT400037285	Chlorophyll a-b binding protein 7, chloroplastic	2.05	2.85
PGSC0003DMT400015075	16kDa membrane protein	1.62	2.77
PGSC0003DMT400071155	Photosystem I subunit XI	2.03	2.72
PGSC0003DMT400092927	Chlorophyll a/b binding protein	1.28	2.69
PGSC0003DMT400049551	Chloroplast chlorophyll a-b binding protein	0.71	2.69
PGSC0003DMT400021871	Chloroplast pigment-binding protein CP29	1.6	2.64

PGSC0003DMT400057281	Chloroplast photosystem I reaction center V	1.87	2.54
PGSC0003DMT400055931	Photosystem II oxygen-evolving complex protein 3	2	2.54
PGSC0003DMT400054836	Chlorophyll a-b binding protein 8, chloroplastic	1.43	2.53
PGSC0003DMT400022698	Chlorophyll a/b binding protein	1.34	2.51
PGSC0003DMT400051932	Photosystem I reaction center subunit IV A isoform 2	1.39	2.5
PGSC0003DMT400019583	PSI-H	2.1	2.48
PGSC0003DMT400092049	Plastocyanin, chloroplastic	1.98	2.46
PGSC0003DMT400035005	Chlorophyll a-b binding protein 3C	1.29	2.45
PGSC0003DMT400075598	PsbP domain-containing protein 3, chloroplastic	1.03	2.43
PGSC0003DMT400068129	Type I (26 kD) CP29 polypeptide	1.29	2.42
PGSC0003DMT400037324	Oxygen-evolving enhancer protein 3-1, chloroplast	1.65	2.41
PGSC0003DMT400037326	Oxygen evolving enhancer 3 family protein	1.8	2.41
PGSC0003DMT400056635	Photosystem I reaction center subunit IV B isoform 2	1.4	2.38
PGSC0003DMT400061952	Fructose-1,6-bisphosphatase, cytosolic	1.51	2.38
PGSC0003DMT400068575	Isoform 2 of PsbP 2, chloroplastic	1.73	2.38
PGSC0003DMT400061951	Fructose-1,6-bisphosphatase, cytosolic	1.55	2.37
PGSC0003DMT400014861	Photosystem I reaction center subunit	1.79	2.36
PGSC0003DMT400021422	Chlorophyll a/b binding protein	1.26	2.36
PGSC0003DMT400019490	Photosystem II reaction center W protein, chloroplastic	1.59	2.32
PGSC0003DMT400075597	PsbP domain-containing protein 3, chloroplastic	1.01	2.32
PGSC0003DMT400034894	Chlorophyll a/b-binding protein PS II-Type I	1.02	2.3
PGSC0003DMT400022699	Chlorophyll a/b binding protein	1.27	2.27
PGSC0003DMT400051899	Photosystem II reaction center W protein, chloroplastic	1.22	2.26
PGSC0003DMT400067194	Thylakoid lumenal 29.8 kDa protein, chloroplast	1.54	2.24
PGSC0003DMT400021390	Chlorophyll a/b binding protein	1.08	2.23
PGSC0003DMT400049395	Fructose-1,6-bisphosphatase	0.77	2.22
PGSC0003DMT400019584	Hydroxyphenylpyruvate reductase	1.94	2.21
PGSC0003DMT400013730	Photosystem I P700 chlorophyll a apoprotein A1	1.14	2.19
PGSC0003DMT400021389	Chlorophyll a/b binding protein	1.07	2.19
PGSC0003DMT400021392	Chlorophyll a/b binding protein	1.37	2.17
PGSC0003DMT400073694	Chloroplast post-illumination chlorophyll fluorescence increase protein	1.14	2.13
PGSC0003DMT400028453	NAD(P)H-quinone oxidoreductase subunit 5, chloroplastic	1.21	2.12
PGSC0003DMT400022700	Chlorophyll a/b binding protein	1.09	2.1
PGSC0003DMT400029489	NAD(P)H-quinone oxidoreductase subunit I, chloroplastic	1.42	2.09
PGSC0003DMT400030012	R2 late blight resistance protein	1.19	2.03
PGSC0003DMT400035006	Chlorophyll a-b binding protein 3C, chloroplastic	0.77	2.03
PGSC0003DMT400019585	Hydroxyphenylpyruvate reductase	1.71	2.03
PGSC0003DMT400019204	Calcium ion binding	1.53	2.01
PGSC0003DMT400019203	Calcium ion binding	1.46	2
PGSC0003DMT400067776	Ferredoxin-3, chloroplast	0.66	0.45

Carbon assimilation is dependent on electron transfer (Wu *et al*. 2008) and several of the transcripts upregulated by BR in this microarray are linked to electron flow/electron carrier activity. This increased photosynthetic capability may be the driving force behind BR-mediated growth.

4.7 Comparison to other published studies

Yin *et al*. (2002) describe microarray analysis of wild type and bes1-D Arabidopsis seedlings treated with 1uM brassinolide, and identify up-regulation in auxin-associated genes such as SAURs, putative expansins, and pectin methylesterases in agreement with the potato transcripts shown to be BR-upregulated in this study. On the other hand, they identify several xyloglucan endotransglycosylases (XETs), cell-wall associated genes, to be upregulated by BR, including XTR6. This is contrary to EBL-treated potato, where 4 out of 5 XETs differentially regulated are down-regulated, with one transcript similar to Arabidopsis XTR6 being down-regulated over 6-fold. However, Mussig *et al.* (2002) show XTR7 to be down-regulated with BR treatment, as is the case in potato transcript PGSC0003DMT400056316 which shows similarity. The XETs are a large class of enzymes, and although they are broadly recognised as growth promoting, there are likely to be differences and specificities in their function.

Sun *et al.* (2010) identify target genes specifically regulated by BZR1. This transcription factor was shown to bind to over 3500 genes, divided into similar numbers of activated and repressed genes. They show BZR1 to positively regulate expansins and pectinesterases as above, again with up-regulation of XETs. In the biotic response category, they show the barley mildew resistance locus O homologue MLO2 to be down-regulated, as well as FLS2. MLO1 is present in the potato microarray data, one of

the top 50 most down-regulated genes at 24h post-EBL, and although the putative potato orthologue of FLS2 (PGSC0003DMT400021384) did not come through as significantly changed in expression in microarray analysis, it has been shown to be down-regulated by BR treatment after 24 hours in qRT-PCR (see **Chapter 5**). BZR1 is shown to upregulate CIPK1, in agreement with potato transcript PGSC0003DMT400011723 being up-regulated more than 2 fold at 24 hours post-EBL treatment. CIPK1 is a signalling gene, linked to ABA signalling, with knock-outs shown to be hypersensitive to osmotic stress (D'Angelo *et al*., 2006). Sun *et al*. (2010) also show that the phytochrome interacting factor PIF3 is down-regulated by BZR1. The potato transcript PGSC0003DMT400047079 has similarity to PIF3, and is down-regulated more than 2-fold at 24h by EBL. However not all trends are in agreement, with Sun *et al*. (2010) showing the blue light photoreceptor PHOT1 shown to be down-regulated by BZR1, but the nearly identical transcript in potato PGSC0003DMT400065251 up-regulated by EBL 2.5 fold. Additionally, the sucrose synthase SUC1 is upregulated by BZR binding, but there are four potato transcripts annotated as sucrose synthases shown to be down-regulated by EBL treatment.

Goda *et al*. (2002) also show the downregulation of PIF3 by BR treatment of Arabidopsis, as well as the upregulation of XETs, expansins, SAURs and pectinesterases. They also describe down-regulation of several genes implicated in BR biosynthesis, such as DWF4 and CPD, as part of the negative feedback mechanism controlling BR homeostasis, with no change in others such as DWF1 or DET2. Notably, no potato orthologue of DWF4 or CPD was found to be differentially regulated by EBL in this study, but two other BR biosynthetic genes with similarity to DET2,

PGSC0003DMT400002569 and PGSC0003DMT400002567 were found to be down-regulated more than three-fold.

4.8 Discussion

These results highlight the importance of regulatory cross-talk between hormone signalling and other signal transduction pathways, with differentially regulated genes linked to ABA, SA, ethylene, auxin, abiotic stress, pathogen challenge and more. Work by Nemhauser *et al*. (2006) analysed microarray data from a variety of hormone treatments in Arabidopsis, and found very little overlap between the gene sets regulated by each, concluding that there is not a core transcriptional growth-regulatory module in the plant. However, their data represents only a 3 hour timecourse. Given the mass of transcriptional change that occurs at 24 hours relative to 3 hours in the data presented here, it may well be that overlap has been largely missed.

Exogenous application of epibrassinolide as used in this study has limitations, and may not accurately mimic brassinosteroid responses in nature. A single spray means the pathway may only be over-activated for only a limited time before returning to basal level (or possibly even lower than basal level for a time as the plant attempts to compensate). In addition, the high concentration of hormone used could stress the plant to an extent and this is likely to affect the outcomes. It would be interesting to sample at more frequent timepoints, and extend the timecourse in order to see how long the effects of exogenous brassinosteroid last. The differences between the significant gene lists at 3 hours and 24 hours in this study highlight the importance of selecting a relevant timepoint when defining BR marker genes. The vast majority of the

genes are only significantly different at one timepoint in this particular microarray, and those that overlap both timepoints do not necessarily show the same trend at each. It would be interesting to compare the effects of differing concentrations of brassinosteroid on the plants, as the relationship between dose and effect may not be a simple linear one. Work by Fridman *et al*. (2014) on root hair and non-hair cells suggests that it is not the absolute level of brassinosteroid that is important, but rather the level relative to nearby tissues. To add further to the complexity, Mussig *et al*. (2003) show BRs to have opposite effects dependent on concentration. Low levels stimulate root growth, but high levels are inhibitory. It would also be prudent to avoid generalising the effects of brassinosteroid in one plant tissue to another, as the response may be very different or even opposite. For example, BR-signalling has been shown to repress stomatal development in cotyledons and leaves (Kim *et al*., 2012), yet increase the number of them in hypocotyls (Fuentes *et al*., 2012). Transgenic potato plants, with attentuated or enhanced brassinosteroid pathway signalling, such as a *bri1* knockouts, or an overactive DWF4, would be a valuable tool for comparing the transcriptome to that of a wild-type potato plant. These would allow the study of the brassinosteroid pathway in potato in a much more stable system, with the level of activity more constant across the experimental timepoints. It would also allow the natural fluctuations in signalling and pathway activity that will occur over the course of a day. Of course these too may not accurately reflect brassinosteroid responses in a natural setting, as the inherent phenotype in brassinosteroid signalling mutants could potentially also further impact on gene regulation. However, a comparison of stable transgenic plants with the exogenous BR application as examined in this study would provide a more reliable picture of core BR-regulated genes. Mussig *et al*. (2002)

describe a robust transcriptomic analysis of BR-regulated genes in Arabidopsis; comparing exogenous BR application, two BR-deficient plant lines, and two different environmental conditions (soil grown and agar medium). The authors note that only a limited subset of genes appear to be truly BR-regulated under all conditions. This reinforces the idea that there is not a clear plant state representing an 'active' or 'inactive' pathway, but rather it is a plastic system sensitive to gradients and inputs from many different sources, and the outcomes are dependent on the integration of all of these. Pokotylo *et al*. (2014) provide a good example of this – while BR has no effect on seed yield in *Brassica napus* under standard growth conditions, it almost doubles the seed yield of plants subjected to salt stress. It may be that some effects of brassinosteroid signalling will be missed unless the plants are under specific physiological conditions at the point of treatment.

The selection of marker genes used in this study was made on the basis of known links to brassinosteroid effects and developmental processes. While this adds weight to their use as markers of BR activity, the data may well contain novel BR-regulated genes or links to novel processes that have been overlooked. This could be particularly relevant to the 28% of genes BR-regulated in this microarray data that have no ascribed function. Valuable information could also be missed by applying the arbitrary 2-fold cut-off to the data during statistical filtering. Although this is standard practice, it may bias the dataset towards genes which are more radically affected by BR treatment, and these might not necessarily be the most relevant. The brassinosteroid pathway has vast impact on the plants physiology, with homeostasis crucial to development. It may be possible that some of the most important genes associated with BR signalling are tightly controlled by more than one mechanism to avoid the

upset of homeostasis, so are in fact changed only modestly by BR application. Yin *et al*. (2002) mention the limited changes in BR-mediated gene expression, commonly within the region of 2-4 fold. This microarray analysis has generated a large amount of data, which warrants further mining to fulfil its potential.

Finally, it is worth noting that the transcriptome may not correlate with the proteome, which is ultimately what will have the most impact on the plants physiology. Deng *et al.* (2007) describe a proteomic analysis of BR-treated Arabidopsis, in which 80% of the proteins affected by brassinosteroid had not been previously identified in microarray analysis. It would be informative to use a proteomics approach to further characterise BR-signalling in potato.

4.9 Conclusions

This microarray analysis investigating the effects of brassinosteroid treatment is the first of its kind to focus on the potato plant *S. tuberosum.* It has provided a glimpse into the breadth of impact that brassinosteroid pathway signalling has on this crop plant; from crosstalk with other hormone pathways such as auxin and ethylene, to the fundamental process of photosynthesis, as well as stress responses, and the growth and developmental processes for which BR is more commonly known. In comparison to published microarray data from *Arabidopsis thaliana* there are clear overlaps, for example the upregulation of SAUR genes and links to auxin, and cell-wall associated genes such as pectinesterases. There are also differences, which could potentially be attributed to plant species, hormone concentration, developmental stage, or environmental inputs such as light, nutrients and temperature.

These results provide a wealth of information that can be taken forward into future studies, and are a starting point in translating what is known about the brassinosteroid pathway in Arabidopsis into the crop species *S. tuberosum*.

CHAPTER 5

PiAVR2 and the trade-off between growth and immunity in *Solanum tuberosum*

5.1 Introduction and Experimental Aims

Work in **Chapter 3** firmly established the role of PiAVR2 in boosting pathogen virulence, and demonstrated active suppression of the plants immune response by this effector. In addition, the experimental evidence suggests that PiAVR2 increases the level, and potentially the activity, of its target protein. Increased StBSL1 levels were observed in the presence of the effector, and overexpression of StBSL1 was shown to result in the same virulence boost and immune suppression as expression of the effector itself. This work was done in the model Solanaceous plant *Nicotiana benthamiana*, with a key next step being to increase the relevance of these findings by examining AVR2 function in the host plant of *P. infestans; Solanum tuberosum*. Experimental work in the following chapter builds upon the transient expression data, and utilises the brassinosteroid pathway marker genes defined from microarray analysis described in **Chapter Four**.

The work in this chapter aims to:

- Investigate the broader impact of PiAVR2 on the host plant *Solanum tuberosum* using transgenic plant lines
- Determine the effect of PiAVR2 on brassinosteroid pathway signalling
- Further knowledge on the crosstalk between the brassinosteroid pathway and immune signalling in plants

5.2 PiAVR2 expression in *Solanum tuberosum*

Whilst Agrobacterium-mediated transient gene expression *in planta* is a tremendously useful tool, stable expression of a gene of interest has advantages, with long-term expression of the transgene providing the opportunity to study effects that would be missed in a transient system. In addition, stable transformation means that after the initial transfection procedure, there is no requirement for the presence of *A. tumefaciens*. Bacterial load has the potential to complicate downstream assays, particularly those which aim to investigate aspects of immunity, as its presence will trigger a degree of immune response in the plant. Additionally, transient expression levels can vary both within the infiltrated site, and between biological repeats, so stable transformation is helpful in removing a variable from the experimental set-up.

To investigate the impact of the effector PiAVR2 on the potato plant, beyond the scale of localised transient expression and cell death assays, stable transgenic lines of *Solanum tuberosum* cv Desiree were generated with constitutive, 35S-promoter driven AVR2 expression by FUNGEN at the James Hutton Institute. Lines were initially screened by RT-PCR for presence/absence of an AVR2 transcript. Of those that passed this screen, five were then analysed for level of AVR2 expression by qRT-PCR (see **Figure 5.1**).

All five lines were shown to express PiAVR2 at levels more than 3-fold that of the housekeeping gene tubulin. Two of these lines, AVR2#29 and AVR2#39, were taken forward for in-depth analysis.

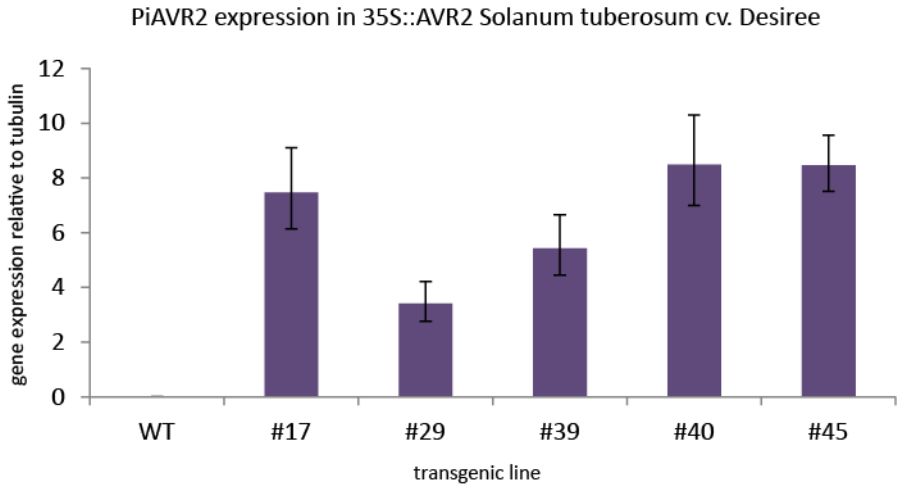

Figure 5.1 PiAVR2 expression in transgenic 35S:PiAVR2 *Solanum tuberosum* lines

qRT-PCR was used to assess expression of the PiAVR2 transgene, with data normalised against and shown relative to the expression of the housekeeping gene tubulin. Leaf material from several 4-week old plantlets was pooled for each line and used to synthesise cDNA. Data represents the average of three technical replicates +/- standard deviation.

Importantly, these lines responded with a clear hypersensitive response when R2 was transiently expressed (**Figure 5.2**). This provides evidence that the AVR2 transgene is translated into a functional protein, and maintains the expected interaction with target StBSL1; facilitating recognition by its cognate resistance protein. *P. infestans* was shown to have improved virulence on these plants, with significantly increased lesion size on leaves when inoculated with sporangia (**Figure 5.3**). This mirrors the results seen with transient expression in *N. benthamiana* described in Chapter 3, confirming that AVR2 is beneficial to the pathogen and increases host susceptibility to disease.

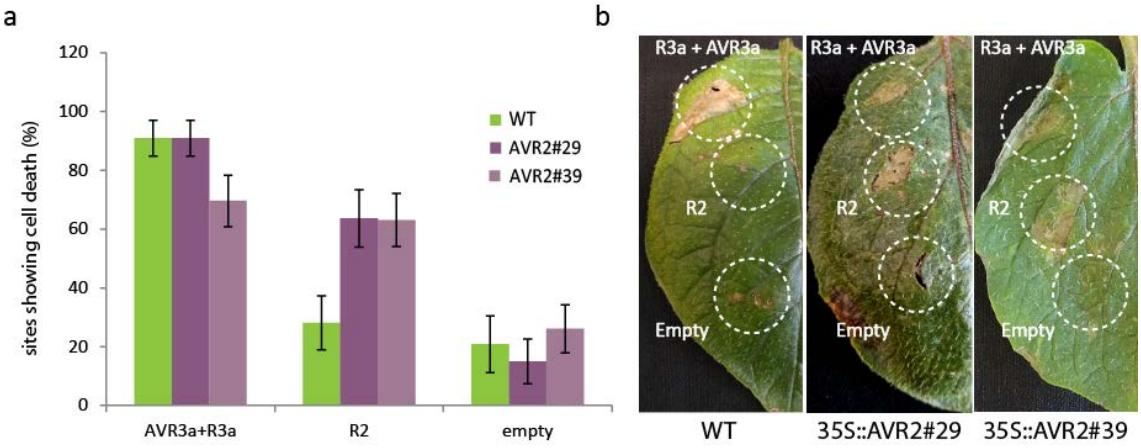

Figure 5.2 R2-mediated hypersensitive response in 35S:PiAVR2 *Solanum tuberosum*

Data collected by Lina Yang (a) Co-expression of PiAVR3a with R3a from *Solanum demissum* resulted in HR at a majority of infiltration sites in WT, #29 and #39 plants. Expression of R2 alone resulted in HR when expressed in #29 and #39 lines, but showed no difference in cell death to an empty vector control when expressed in WT leaves. (b) Representative leaf images showing HR cell death as depicted in (a).

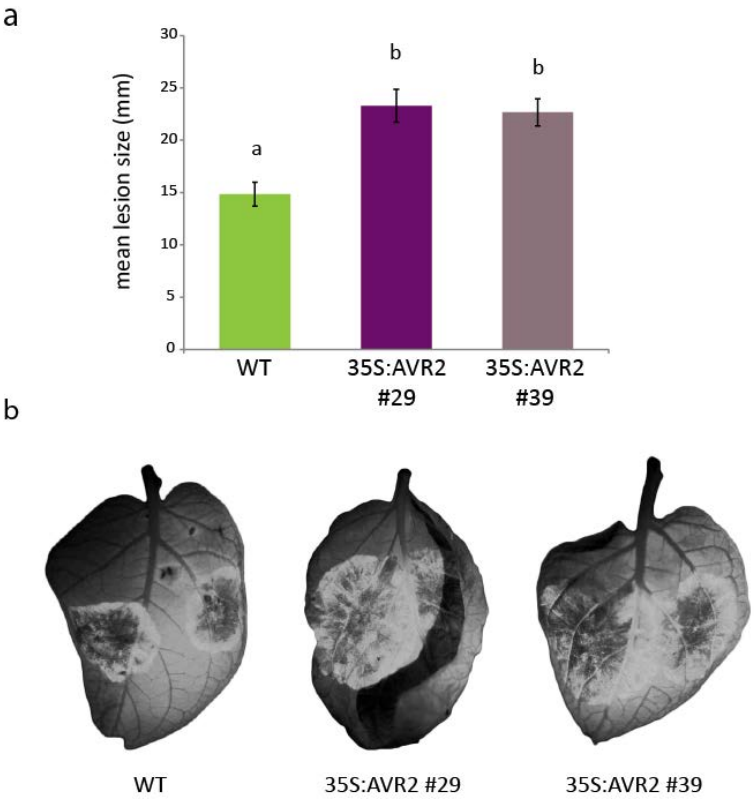

Figure 5.3 *P. infestans* leaf colonisation in 35S:PiAVR2 *Solanum tuberosum*

(a) *P. infestans* lesion size (diameter in mm) on 35S:AVR2 potato at 7 days post inoculation of sporangia suspension. Error bars represent SEM, a≠b $p<0.001$ (one-way ANOVA with Holm-Sidak). (b) Representative leaf images showing increased *P. infestans* lesion size on 35S:AVR2 potato compared to untransformed WT potato. Images were taken under UV light.

A phenotype of 35S:AVR2 expression became apparent in plantlets grown in petri dishes, with leaves curling downwards at the edges. Root growth on plates was also notably weaker in the transgenic lines compared to WT plants. When plantlets were transferred to soil, the developmental differences became even more striking, with 35S::AVR2 plants exhibiting a twisted, spiralling growth habit with curled petioles and leaves. Leaves lost their usual symmetry, with reduced number of leaflets per compound leaf. Upon closer inspection, these plants also had a variety of other more subtle characteristics, such as thickening of trichomes and anthocyanin accumulation, as well as fused leaflets in some cases. These phenotypes can be visualised in **Figure 5.4**. The plants were challenging to work with; clearly stressed, often exhibiting early senescence, and frequently succumbing to infection both in tissue culture and in glasshouse conditions.

The growth habit of these plants, with exaggerated twisting of stems and leaves, is broadly reminiscent of BR-overactive mutants in Arabidopsis, such as those with BRI1 and DWF4 overexpression (Wang *et al*., 2001) and bik1 mutation (Lin *et al*., 2013). The fusion of leaflets in the 35S:AVR2 plants bears similarity to the organ fusion phenotypes seen with BZR1 overexpression (Gendron *et al*., 2012), attributed to BR signalling resulting in reduced expression of CUC family genes which are responsible for determining organ boundaries. Several studies have linked the activity of BR signalling to root development, with low levels (<1nM) stimulating root growth, but high levels (>1nM) inhibiting root growth in Arabidopsis (Mussig *et al*., 2003; Clouse *et al*., 1996). Anthocyanin levels are known to positively

Figure 5.4 Phenotype of PiAVR2-expressing *Solanum tuberosum*

(a) shows the full extent of the developmental phenotype of two transgenic lines of potato expressing PiAVR2 (#29 and #39). (b) depicts a wild-type leaf, with (c) showing a 35S::AVR2 line with increased trichome thickness in comparison. (d) shows leaf formation of wild-type compared with 35S::AVR2 plants, with compound leaf formation adversely affected. (e) shows increased colouration of the leaf underside in the transgenic plants, characteristic of anthocyanin accumulation. (f) is an aerial view of AVR2#29 to highlight a characteristic 'spiralling' growth habit, with (g) showing strong leaf curling in a young plant. (h) depicts fusion between leaflets within the compound leaf in AVR2#39.

correlate with brassinosteroid signalling. Symons *et al*. (2006) describe the promotion of ripening in grape (*Vitis vinifera*) by application of brassinosteroid, with BR treatment shown to increase the expression of several genes involved in anthocyanin biosynthesis (Peng *et al*., 2011; Yuan *et al*., 2014). This is also supported by the upregulation of several anthocyanin-related transcripts in the microarray, described in **Chapter 4**. Trichome thickening does not appear to be directly linked with increased BR signalling, but has been associated with increased gibberellic acid (GA) signalling (Perazza *et al*., 1998). This plant hormone has many developmental effects that overlap with those of BR signalling (Bai *et al*., 2012), and brassinosteroid signalling is recognised as a key regulator of GA biosynthesis in plants (Unterholzner *et al*., 2015). The observed early senescence of the 35S:AVR2 plants may be attributable to brassinosteroids, with He *et al*., (1996) showing accelerated senescence of mung bean leaves after exogenous BR treatment. This is complemented by the delayed senescence of BR-deficient plants, such as the Arabidopsis *dwf1* mutant (Choe *et al*., 1999) and *det2* mutant (Wang *et al*., 2001).

Differences between wild-type *Solanum tuberosum* and 35S::AVR2 plants were also observed at the microscopic level, with stomata appearing larger and fewer in number. Epidermal cells were observed to be larger (**Figure 5.5**). To quantify stomata whilst taking the number of epidermal cells into account for a given area, stomatal density was calculated. This revealed a decreased stomatal density in the 35S::AVR2 plants, from 25% to approximately 20% stomata (**Figure 5.5**). This small yet statistically significant decrease corresponds with data published by Kim *et al*., (2012), showing that overexpression of BSL family members from Arabidopsis decreases the percentage of stomata to a similar degree. The authors complement these findings by

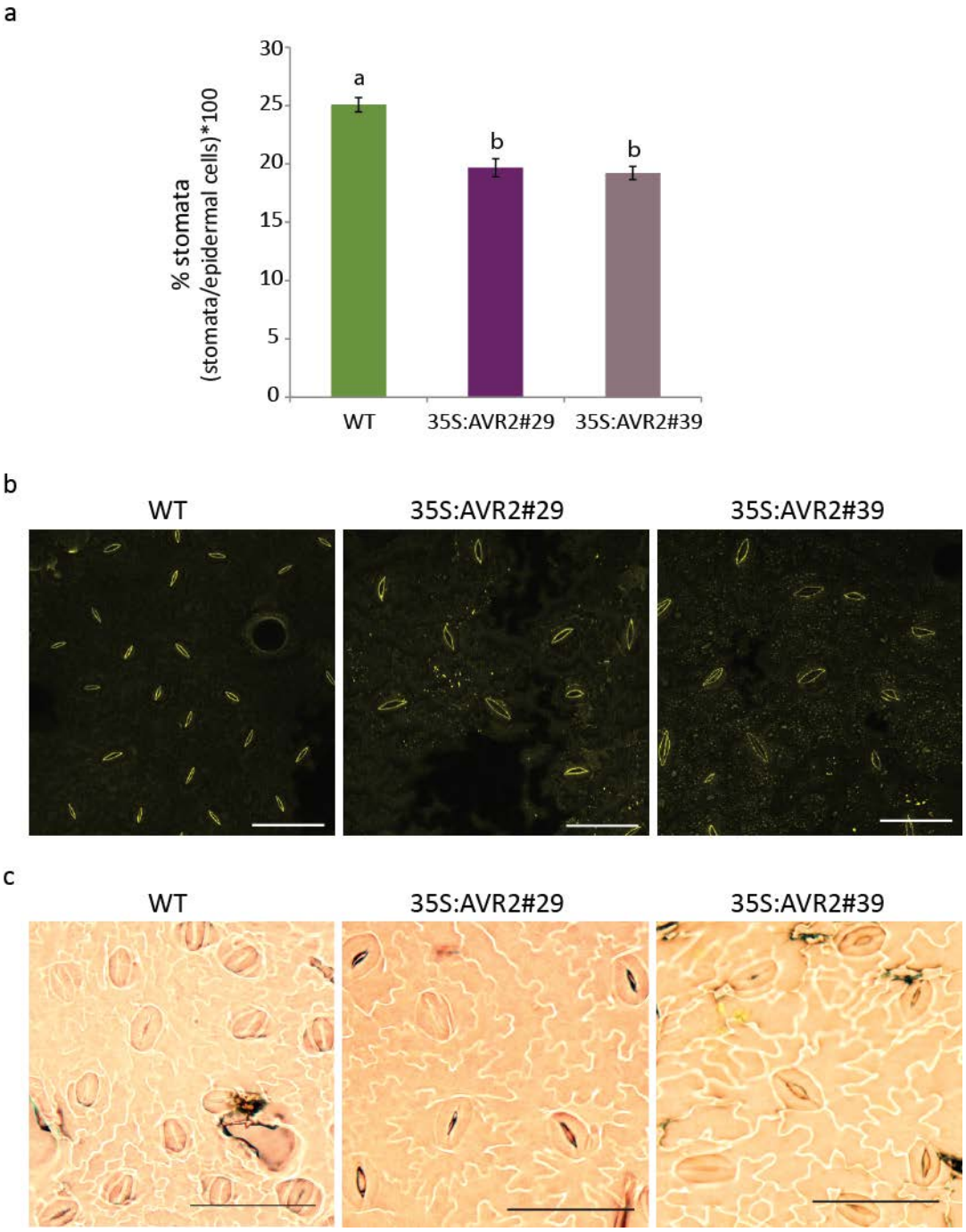

Figure 5.5 Reduced stomatal density in 35S:AVR2 *Solanum tuberosum*

(a) Reduced percentage of stomata in 35S:AVR2 potato plants. Stomata count was expressed as % of total epidermal cells counted per 500 µm. Results combine three biological replicates, each consisting of epidermal leaf prints from three or more plants. Error bars indicate SEM; letters denote significant difference ($p<0.001$ in one-way ANOVA, Holm-Sidak). (b) Confocal microscopy showing reduced stomatal frequency in 35S:AVR2 potato, and enlargement of stomata relative to WT plants. Images are of representative leaves stained with calcufluor white. Scale bar = 100µm. (c) Bright-field microscopy of epidermal leaf prints depicting enlarged epidermal cells in 35S:AVR2 potato, compared to WT. Black line size markers indicate 100µm.

showing that *bsu-q* (a quadruple knock-out mutant for AtBSU1, BSL1, BSL2 and BSL3) exhibits extreme stomatal clustering, as does the BR biosynthetic mutant line *det2-1*. Additionally, Kim *et al*., (2012) show a clear reduction in stomatal density in plants grown on media containing brassinolide, and conclude that brassinosteroid pathway signalling inhibits stomatal development. In the context of the 35S:AVR2 transgenic potato plants, this suggests that presence of the effector has increased levels of the BSL family proteins, resulting in increased brassinosteroid pathway activity and the consequent repression of stomatal development.

5.3 PiAVR2 and the brassinosteroid pathway

Several lines of evidence now point towards PiAVR2 over-activating the brassinosteroid pathway in potato: firstly, the observation that BSL1 over-expression mimics that of the effector itself; suppressing INF1 cell death and boosting pathogen virulence. Secondly, the stabilisation of the BSL proteins by AVR2 when transiently expressed. Thirdly, the phenotype of 35S:AVR2 plants, reminiscent of BR overactive mutants. To confirm that this was the case, marker genes for BR pathway activity as discussed in Chapter 4 were used in qRT-PCR, to compare expression levels between wild type and 35S::AVR2 plants (**Figure 5.6**). All five BR-induced marker genes (StEXP8, StHBI1-like, StP69F, StCAB50, and StSAUR are strongly upregulated in the 35S::AVR2 plants, with the BR-repressed marker, StGA2ox1, being strongly down-regulated. This revealed the striking observation that PiAVR2 expression in the plant recapitulates the effect of EBL treatment, leading to the conclusion that these plants do indeed have increased activity of the brassinosteroid pathway. Not only is BR pathway activity increased, but the extent of this increase appears to be extreme – the transcriptional

changes are almost identical to those seen with 50µM EBL treatment for several of the marker genes. This represents a very high concentration, with concentrations of as little as 1nM shown to have a stimulatory effect on growth (Kim *et al*., 2007).

Further, the 35S:AVR2 plants were also shown to have reduced basal expression levels of three PTI marker genes; StWRKY7, StACRE31, StCYP71D20, in addition to reduced expression of the flagellin receptor StFLS2 (**Figure 5.7**). Together these results provide a potential mode of action for the *P. infestans* effector AVR2; enhancing brassinosteroid pathway signalling in order to exploit crosstalk between growth and immunity, thus indirectly suppressing the host plants immune response.

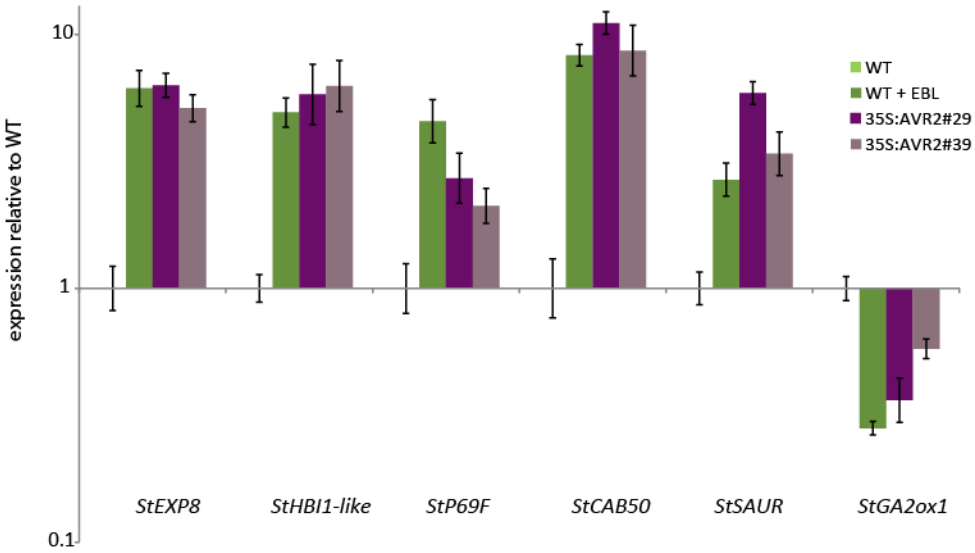

Figure 5.6 Expression of brassinosteroid pathway marker genes in 35S:AVR2 *Solanum tuberosum*

Relative expression of brassinosteroid-regulated genes in untreated potato cv. Desiree (WT; given a value of 1), WT at 24 h after treatment with EBL; and constitutive levels of expression in 35S:AVR2 potato plants, assessed by qRT-PCR. Expression was normalised to *StUbi* and shown relative to WT untreated plants. Data represents the average of three technical replicates +/- standard deviation, with similar patterns of expression observed in at least 2 biological replicates.

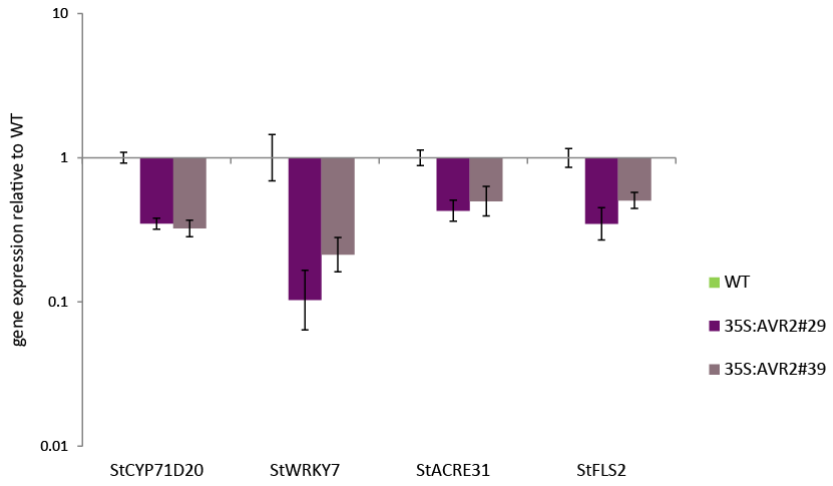

Figure 5.7 PTI marker genes downregulated in 35S:PiAVR2 *Solanum tuberosum*

Relative basal expression of four PTI-associated genes in 35S:AVR2 potato compared to WT, assessed by qRT-PCR. Expression was normalised to StUbi and shown relative to WT plants. Data represents average of three technical replicates +/- standard deviation. A similar pattern in gene expression was observed in at least two biological replicates. Data collected in collaboration with Lina Yang.

5.4 StHBI1-like as a negative regulator of immunity

While the negative effect of BR signalling on PTI is now widely accepted, the actual mechanism is not well understood. How does brassinosteroid signalling inhibit the plants first layer of defence? Initial studies focused efforts at the start-point of these signalling pathways, on BAK1 – a co-receptor shared by both BRI1 and FLS2 among other PRRs, and therefore a logical place to look for crosstalk. Belkhadir *et al.* (2012) show that overexpression of BRI1 can suppress the plant immune response to flg22, elf18 and peptidoglycans, and that this is remedied by the concurrent overexpression of BAK1, suggesting that BAK1 is rate-limiting in the immune response. However Albrecht *et al*. (2012) show that this is not the full picture, with BR treatment able to suppress chitin-mediated immune signalling despite the fact that BAK1 is not involved

in chitin perception. Malinovsky *et al*. (2014) examined the point of crosstalk between BR signalling and immunity, and found it to be at the level of BR-induced transcriptional regulation, much further down the pathway than was originally postulated. As described in the introduction, perception of BR triggers a signalling cascade driven by phosphorylation and dephosphorylation of its subunits, and culminates in the activation of two transcription factors, BES1 and BZR1 (Kim *et al*., 2011; Clouse, 2011) which are master regulators of BR-responsive genes. BZR1 activation was shown by Lozano-Duran *et al*. (2013) to inhibit PTI responses, with an over-representation of defence-related genes under its transcriptional control. Several of these are WRKY transcription factors, for example WRKY11, 15 and 18, which are up-regulated by BR and act as negative regulators of PTI. The BZR1-regulated bHLH transcription factors HBI1, CIB1 and BEE2 are also implicated in the negative regulation of PTI by Malinovsky *et al*. (2014); shown to negatively regulate immunity, and additionally shown to be down-regulated upon PAMP perception.

An exciting discovery in the set of BR-upregulated genes identified in Chapter Four was the transcription factor StBHLH7 – a putative potato orthologue of AtHBI1, and as such, re-named StHBI1-like in this study. This was one of the most strongly up-regulated genes by BR treatment of potato, with expression 13-fold higher than untreated. However, the relatively low 42% identity between AtHBI1 and StHBI1-like raised the question of whether this potato protein was functionally equivalent. To investigate this, StHBI1-like was cloned into plant expression vectors for Agrobacterium-mediated transient expression, to be used for INF1 cell death assays and *P. infestans* colonisation assays (**Figure 5.8**). StHBI1-like was shown to significantly suppress INF1 cell death, reducing positive HR sites by around 50%. This mimics the

results seen with PiAVR2 and StBSL1 transient expression described in Chapter Three. In addition, StHBI1-like provided a significant boost to *P. infestans* virulence on *N. benthamiana*, with average lesion size almost doubled by transient expression of the transcription factor.

To examine the effects of StHBI1-like at the level of transcriptional regulation, transient expression in *N. benthamiana* was combined with culture filtrate treatment to induce PTI responses. QRT-PCR was then used to compare induction of two PTI marker genes; NbWRKY7 and NbACRE31 (Ishihama *et al*., 2011; Nguyen *et al*., 2010). Transient expression of PiAVR2 and StBSL1 were also included for comparison. **Figure 5.9** shows clear suppression of NbWRKY7 induction by PiAVR2, StBSL1 and StHBI1-like, resulting in less than 10-fold upregulation compared to 25-fold upregulation after culture filtrate in the empty vector control. NbACRE31 induction is also suppressed, although to a lesser extent.

These results support the identification of StHBI1-like as a negative regulator of immunity in Solanaceous plants, and also provide a clearer picture of PiAVR2 in context; stabilising its host target StBSL1, resulting in increased BR-pathway signalling which up-regulates the transcription factor StHBI1-like; culminating in the suppression of PTI.

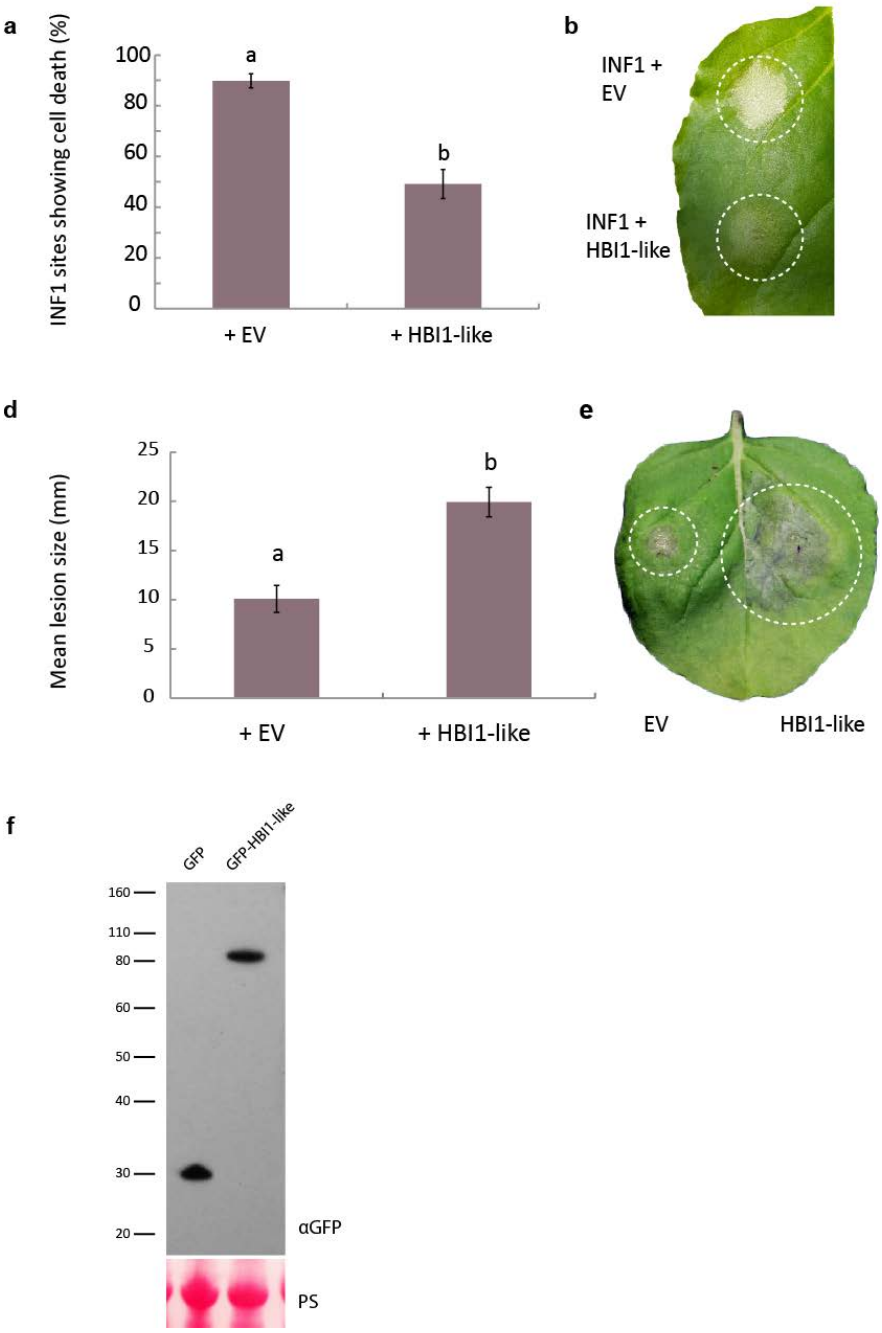

Figure 5.8 StHBI1-like suppresses INF1 cell death and boosts *P. infestans* virulence

(a) Graph shows percentage of leaf infiltration sites at 5 dpi resulting in cell death following *Agrobacterium*-mediated co-expression of INF1 with either HBI1-like or an empty vector (EV) control. Error bars show SEM, a≠b (p≤0.001) in one way ANOVA (Holm-Sidak). Results are combined from 4 biological replicates consisting of at least 4 plants, each with at least 6 infiltrations per plant per expression combination. (b) Representative leaf image showing suppression of INF1 cell death when HBI1-like is co-expressed. (c) StHBI1-like or an empty vector control were transiently expressed in *N. benthamiana*. Sites were inoculated with *P. infestans* sporangia suspension 24 hours later, with lesions measured (diameter in mm) at 7dpi. Error bars show SEM; letters denote significant difference (p≤0.001 in one way ANOVA, Holm-Sidak). Results are combined from 4 biological replicates. (d) Representative leaf image showing increased *P. infestans* colonisation following StHBI1-like expression in *N. benthamiana*. Data collected in collaboration with Lina Yang.

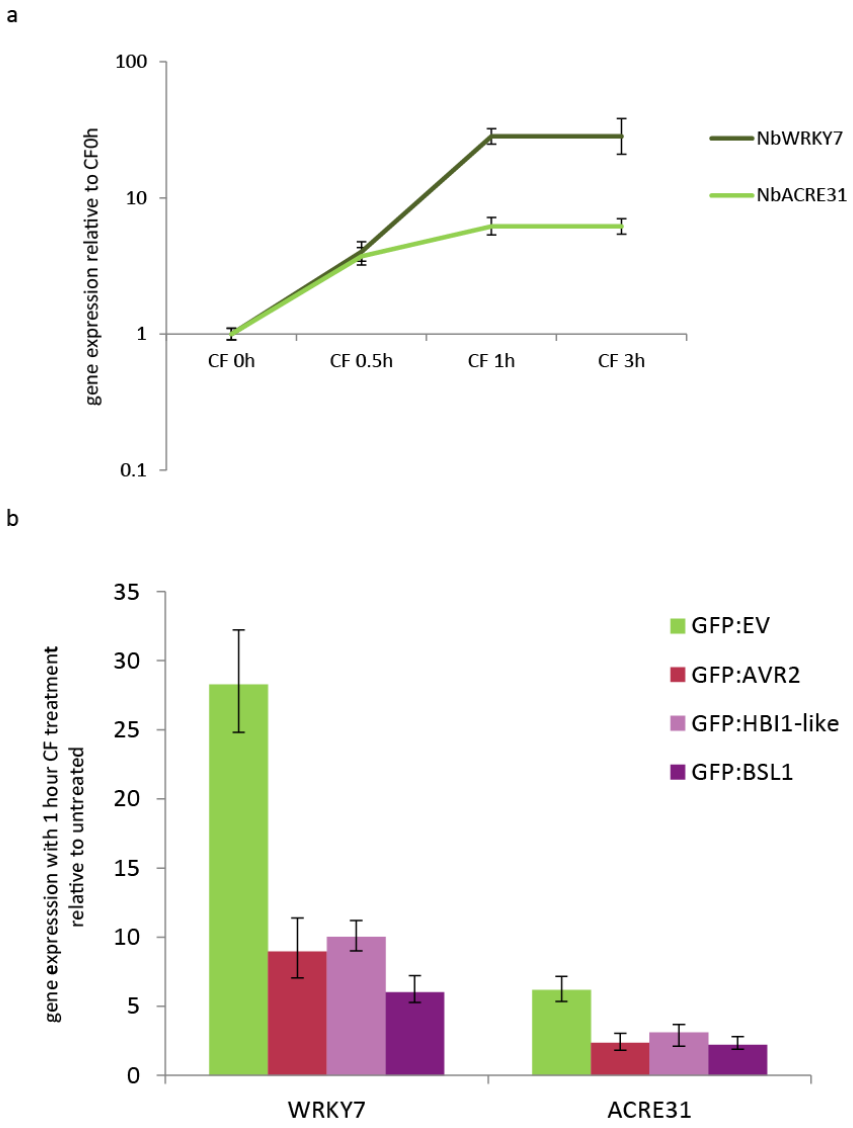

Figure 5.9 PiAVR2, StBSL1 and StHBI1-like suppress induction of PTI marker genes in *N. benthamiana* with culture filtrate treatment
(a) Expression of PTI marker genes NbWRKY7 and NbACRE31 in a culture filtrate timecourse, showing peak expression is reached 1 hour post treatment. (b) Expression of NbWRKY7 and NbACRE31 1 hour post culture filtrate treatment in leaves transiently expressing PiAVR2, StHBI1-like, StBSL1 or empty vector (EV) control. Graphs represent average of three technical replicates in qRT-PCR +/ standard deviation, with similar patterns observed in two biological replicates. Data collected in collaboration with Lina Yang.

5.5 Reciprocal antagonism between the brassinosteroid pathway and PTI signalling

The negative impact of BR signalling on PTI has become well established in recent years, supported by a range of literature as discussed in **Chapter 1**. This was described

as unidirectional; with immune signalling shown to have no effect on BR pathway activity (Albrecht *et al.,* 2011, Belkhadir *et al.,* 2012). To investigate this in the context of the brassinosteroid pathway in potato, qRT-PCR was used to assess the expression of BR-induced genes in plants undergoing an immune response (**Figure 5.10**). These genes, StHBI1-like, StEXP8, StSAUR, and StCAB50 were taken from the set of BR markers identified and validated by the microarray work described in Chapter Four. Two verified marker genes of active PTI, StWRKY7 and StACRE31, were used as positive controls. PTI was induced both with the bacterial flagellin peptide flg22, to induce immune signalling specifically via the receptor-like kinase FLS2, and additionally with *P. infestans* culture filtrate. Culture filtrate represents a 'PAMP cocktail', containing multiple proteins secreted by the pathogen into liquid media during culture that are capable of eliciting an immune response. Strikingly, while PTI markers are up-regulated as expected, there is clear active downregulation of BR pathway markers. This challenges the previously published results from work on *Arabidopsis thaliana*, and represents a novel mode of regulating resource allocation in the plant. In addition, the expression of two genes associated with BR biosynthesis, StDWF5 and StSTDH, were examined during these timecourses (**Figure 5.11**). The results show clear downregulation of these genes, which may provide the mechanism for the antagonism of BR signalling shown. If biosynthesis of brassinosteroid is impaired during the immune response, there will be reduced BR perception, thus less BR pathway activation and consequently the observed downregulation of BR-induced genes. During the preparation of this manuscript, work published by Jiménez-Góngora *et al*. (2015) shows support for these findings, demonstrating downregulation of multiple BR-biosynthetic genes in Arabidopsis when plants were treated with a variety of PAMPs.

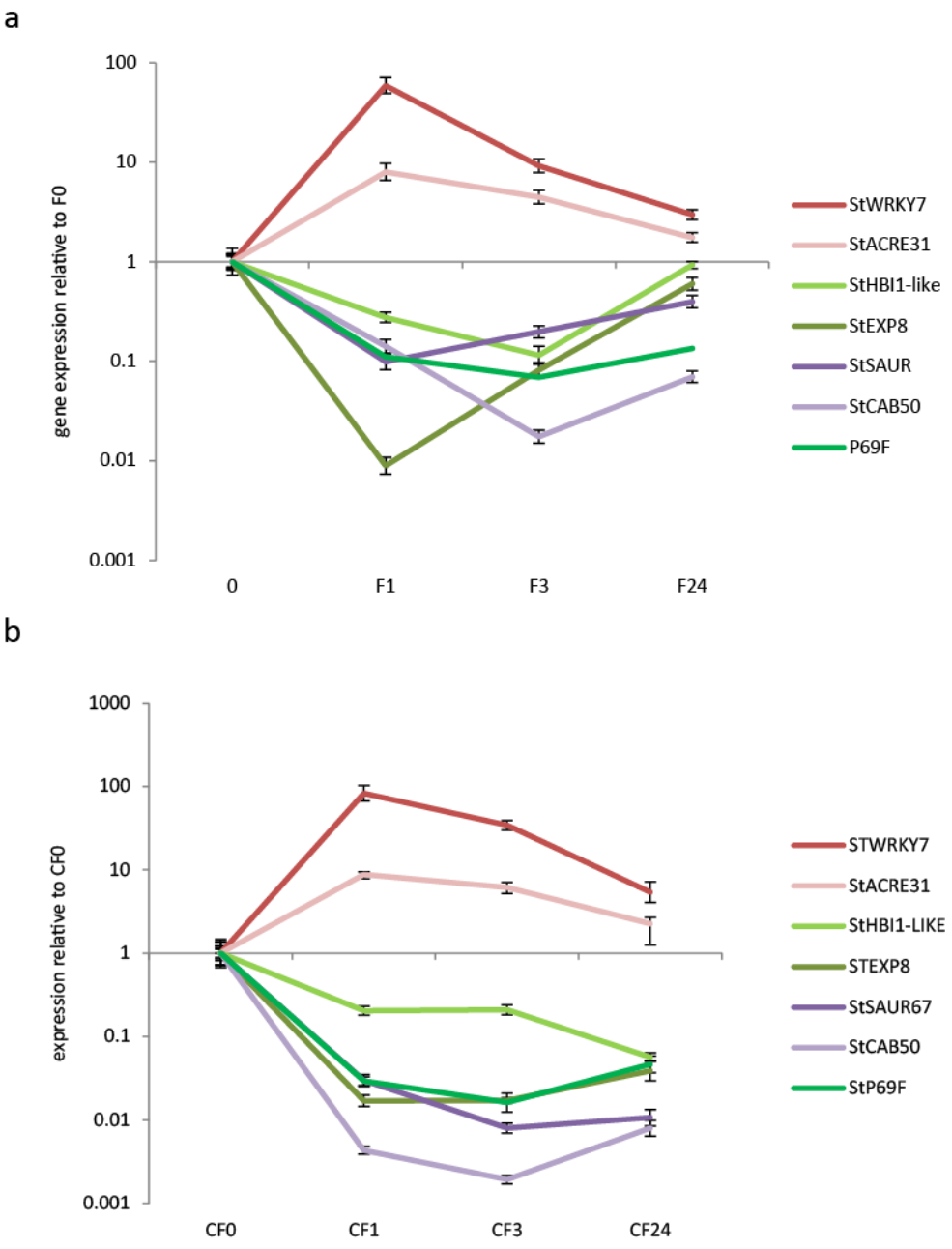

Figure 5.10 Suppression of BR marker genes by PAMP treatment

(a) Treatment of potato cv. Desiree with *P. infestans* culture filtrate (CF) results in transcript accumulation of PTI marker genes *StWRKY7* and *StACRE31* by 1 hour after treatment (CF1h), but reduced transcript abundance of BR (EBL)-induced genes *StHBI1-like, StEXP8, StSAUR, StCAB50* and *StP69F*. (b) Treatment of potato cv. Desiree with the bacterial PAMP flg22 results in similar, opposing patterns of transcript abundance by 1 hour after treatment (F1h) for PTI, and BR markers as observed in (a). Data represents average of three technical replicates +/- standard deviation, with similar patterns of expression observed in at least two independent biological replicates. Data collected in collaboration with Lina Yang.

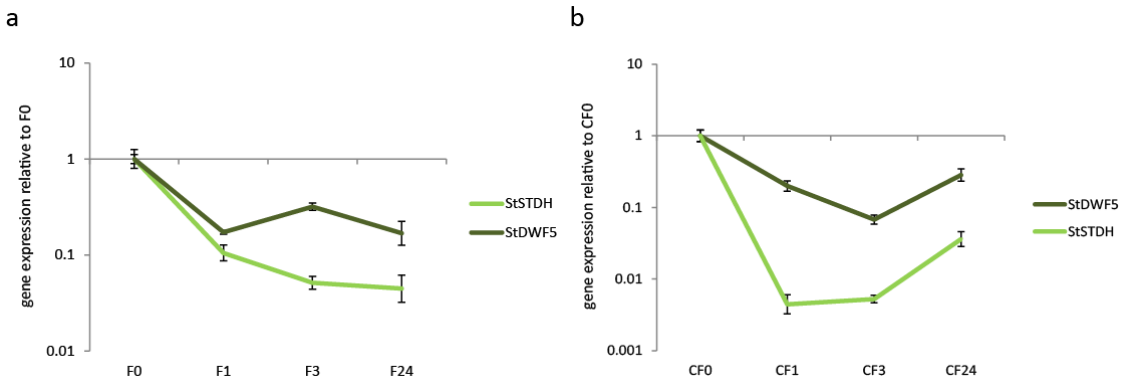

Figure 5.11 Downregulation of BR biosynthethic genes in PTI response

Treatment of *Solanum tuberosum* cv. Desiree with (a) flg22 and (b) *P. infestans* culture filtrate results in down-regulation of two BR-biosynthetic genes StSTDH and StDWF5, with effects persisting for 24 hours. Data represents average of three technical replicates in qRT-PCR +/- standard deviation, with similar patterns of expression observed in at least two independent biological replicates. Data collected in collaboration with Lina Yang.

5.6 Discussion

Work in this chapter aimed to examine the effect of PiAVR2 on *P. infestans'* host plant *Solanum tuberosum*, with the striking discovery that the pathogen is able to manipulate a key hormone signalling pathway in the plant; the brassinosteroid pathway. PiAVR2 increases BR pathway activity, exploiting the crosstalk between it and PTI signalling, leading to the attenuation of plant defences. Further, this work has identified a key negative regulator of immunity in potato, StHBI1-like. This bHLH transcription factor is under opposing regulation by BR and PTI – upregulated by BR treatment, but down-regulated during an active immune response, and shown to suppress aspects of PTI whilst boosting *P. infestans* infection.

The 35S:AVR2 potato plants, despite being difficult to work with due to frequent infection and early senescence, represent a tool that could be exploited further to

learn more about AVR2 function. One interesting aspect would be to examine the levels of BSL family members, to confirm if indeed AVR2 increases their stability. Although this was studied in *N. benthamiana* using transient over-expression of PiAVR2 and GFP-tagged BSLs, assaying endogenous BSL proteins would be more physiologically relevant. This would require antibodies specific to the BSLs, which were obtained during the course of this work but unfortunately proved difficult to optimise, with high background obscuring any specific signal from the BSLs.

It would be highly relevant to assay the growth of other pathogens on these plants, beyond *P. infestans*. Whilst the reduced induction of PTI genes suggests that immunity in general is suppressed to an extent, it would be interesting to see if this translates to increased susceptibility to other pathogens. In particular, it would be prudent to compare the success of biotrophs/hemibiotrophs with necrotrophs on these plants. The defence mechanisms against these two pathogen lifestyles are largely considered to be antagonistic, with the SA-driven defence against biotrophs compromising the ET/JA signalling associated with defence against necrotrophs and vice versa (Glazebrook, 2005). Brassinosteroids have been positively linked to JA biosynthesis and signalling (Mussig *et al*., 2000; Yang *et al*., 2013), whilst shown to have negative impact on SA-mediated immunity (De Vlesschauwer *et al*., 2012).

A key experiment, unfortunately not included in this study, would be to assess the expression of these BR marker genes during a *P. infestans* infection timecourse on potato. Natural levels of PiAVR2 in an infected plant are likely to be much lower than the 35S driven expression in the transgenic plants, but it would be hoped that a positive impact on BR signalling could still be observed, albeit more modest than that

seen in the 35S:AVR2 plants. It may also prove to be the case that AVR2 is not the only effector protein from *P. infestans* that manipulates this hormone signalling pathway to benefit pathogenicity; indeed, there may be multiple effectors across many pathogen species that achieve this in some manner. To assess this, an infection timecourse with *P. infestans* silenced for AVR2 would be useful, to compare BR marker gene expression both with and without this effector.

A fascinating additional discovery made in the course of this work is the evidence that BR antagonism of PTI is not unidirectional, as was thought previously. Albrecht *et al.* (2011) show no change in BR-induced BES1 dephosphorylation when combined with flg22 treatment, and show that BR-induced down-regulation of CPD expression is also unaffected. Belkhadir *et al*. (2012) use BR-induced BKI1 membrane dissociation to assess any effects of PAMP signalling on BR pathway activity, and find no effect. Both sets of authors conclude that flg22 signalling has no effect, positive or negative, on BR signalling. However, both of these studies rely on exogenous BR treatment combined with PAMP treatment, which is likely to have masked the true effect of PTI on the BR signalling readout. If PTI exerts its negative effects by reducing BR biosynthesis and thus BR levels, then replacing this with exogenous hormone will negate the antagonism. Jimenez-Gongora *et al.* (2015) report the presence of binding sites for defence-induced transcription factors in the promoter regions of BR biosynthetic genes, which may provide the mechanism for this cross-talk. Reciprocal antagonism between the brassinosteroid pathway and immune signalling is an elegant way of controlling the allocation of resources in the plant, ensuring that finite reserves are deployed where they are needed most.

5.7 Conclusions

Work in this chapter has confirmed the up-regulation of brassinosteroid pathway signalling by the *P. infestans* effector AVR2, with BR-regulated genes showing similar trends in expression in both EBL-treated and 35S:PiAVR2 *Solanum tuberosum*. Accordingly, 35S:PiAVR2 plants exhibit a phenotype strongly reminiscent of BR-overactive mutants in the literature, and permit increased lesion size of the pathogen. This represents a stealthy mode of immune suppression by a pathogen, and novel function of an oomycete effector protein.

Additionally, this work has identified a negative regulator of immunity in potato; the BHLH transcription factor StHBI1-like. This begins to unravel the complexities of cross-talk between growth and development and the immune response in Solanaceous plants, and is a fascinating example of a pathogen exploiting the plants endogenous negative regulation of defences.

CHAPTER 6

General Discussion and Future Perspectives

This work aimed to determine the function of PiAVR2 in *P. infestans* virulence, by investigating the role of the StBSL protein family as host targets, as well as examining aspects of effector recognition by the plant resistance protein R2. The interplay between the brassinosteroid signalling pathway and the inducible immune response in Solanaceous plants was also examined, with the discovery that *P. infestans* appears to be manipulating this crosstalk to increase its virulence potential.

Elucidating the role of the effector PiAVR2 in potato late blight goes a small way towards understanding the plant-microbe interactions that underpin one of the most significant crop diseases in history. PiAVR2 is one effector protein of potentially hundreds secreted by the pathogen during the infection process, and much remains to be discovered. However this work reveals a novel virulence strategy in effector biology, with potential relevance across a variety of pathogens and crop species.

Chapter Three established StBSL1, one of the host target proteins of PiAVR2, as a susceptibility factor; a host protein which has a positive effect on pathogen virulence. StBSL1 is capable of increasing *P. infestans* virulence, and can function as an immune suppressor, reducing INF1 cell death when co-expressed. StBSL1 was mutated at the predicted active site of the phosphatase domain, reducing the recognition of PiAVR2 by the resistance protein R2, thus implicating phosphatase activity in the R2-mediated HR. Two other members of the StBSL family; BSL2a and 2b, were also studied, with the

intriguing finding that StBSL2b behaves in a contradictory manner to StBSL1 and 2a; actively increasing INF1 cell death and suppressing the AVR2-R2 HR.

Chapter Four describes analysis of EBL-treated potato by microarray, to identify genes up and down-regulated by this hormone. This microarray analysis provided an overview of the vast impact of brassinosteroid treatment on the potato plant, with over 2000 transcripts differentially expressed. These span a breadth of roles including photosynthesis, hormone crosstalk, stress responses, and growth and development, with particularly strong enrichment of genes associated with signalling. This data set enabled the validation of a set of marker genes that can be used to assess BR pathway activity specifically in potato.

Chapter Five characterises transgenic *Solanum tuberosum* cv. Desiree which was modified to express PiAVR2. These plants display elements of a BR-overactive phenotype, with a striking twisted growth habit, and several more subtle changes such as decreased stomatal density. As predicted, these were more susceptible to *P. infestans*, supporting a role of PiAVR2 in pathogen virulence. The plants were confirmed to have upregulated brassinosteroid signalling, determined by gene expression analyses using the BR marker genes identified in **Chapter Four**. One of these BR-upregulated markers was StBHLH7, renamed StHBI1-like in this work. This gene was found to have high sequence similarity to HBI1 and CIB1 in Arabidopsis; transcription factors recently identified as suppressors of PTI by Malinovsky *et al.* (2014). This study identified a similar role for StHBI1-like in Solanaceous plants, with overexpression resulting in the suppression of INF1-mediated cell death, and increased

P. infestans lesion size in leaf inoculations. This transcription factor was also shown to suppress the induction of PTI marker genes upon culture filtrate treatment in *N. benthamiana*, to a similar extent as PiAVR2 and StBSL1 overexpression. Additionally, this work identified reciprocal antagonism between the BR pathway and immune signalling, as well as potentially linking this to the downregulation of BR biosynthesis upon induction of the immune response. This crosstalk was previously considered to be unidirectional, but the significant suppression of several BR-induced genes by PTI induction suggests that this is not the case.

6.1 Mechanism for immune suppression by PiAVR2

The research described in this work contributes considerably to what is known about the function of the effector AVR2 in *P. infestans* virulence. It has been determined that host immunity is suppressed by the effector, indirectly, by exploiting the crosstalk between growth and development and the plant immune system. However, one crucial aspect remains unsolved; the molecular mechanism by which this is achieved. What does PiAVR2 actually do to its interacting proteins StBSL1, StBSL2a and StBSL2b, to result in over-active brassinosteroid pathway signalling and subsequent host immune suppression? The recent discovery that oligomerisation occurs between these family members in Arabidopsis via a 'KKVI' motif (Kim *et al*., 2015) has provided an exciting new avenue to explore, and ongoing work on the BSL protein family in potato is beginning to reveal the finer details of their interaction and that with PiAVR2. The interaction between these proteins, and the differing outcomes from their individual overexpression, are suggestive of a possible regulatory role between family members. Phosphatases, in contrast to kinases, are relatively few in number and largely lacking in

specificity. Where kinases have increased specificity by gene duplication and specialisation, phosphatases have achieved diversity by interacting with a large number of regulatory subunits, forming 'holoenzymes' (Hendrickx *et al*., 2009). These interactors can specify substrate or localisation, act as inhibitors or chaperones, or achieve combinations of these roles (Wakula *et al.*, 2003). This could provide the biological explanation for BSL oligomerisation – they each may act as regulatory elements of the other family members, as well as having their own function regulated in a similar manner. They may be phosphatase regulators as well as phosphatases.

The KKVI motif shown to facilitate BSL oligomerisation in Arabidopsis can be found in StBSL2, with StBSL1 and StBSL3 possessing a similar KKII and KKLI respectively. The online motif prediction tool 'Motif' (http://www.genome.jp/tools/motif/) predicts this region to be the start of a serine-threonine protein phosphatase N-terminal domain; an important region that influences the properties of the phosphatase (Xie *et al.*, 2009). Also of note is another similar motif approximately 160 residues downstream, consisting of KKII which is conserved across all three StBSLs (Shown boxed in **Figure 6.1**). These motifs bear similarity to the PP1 docking motif 'RVxF', a region frequently found in proteins that interact with PP1 either as regulatory subunits, or directly as substrates in some cases. Proteins with this motif can bind to the hydrophobic groove of PP1, without directly affecting enzymatic activity *per se*, as the active site is out-with this region (Peti *et al.*, 2013).

This loosely conserved 'RVxF' motif is actually a degenerate motif consisting of 4 or 5 residues [R/K]-X_{0-1}–[V/I]-{P}-[F/W], describing Valine or Isoleucine separated from

Phenylalanine or Tryptophan by any residue except Proline, with Arginine or Lysine in at least one of the two preceding positions (Wakula *et al*., 2003). However, the F/W residue (which would provide a hydrophobic side chain) is lacking in the BSL sequences. The downstream KKII motif, although also lacking the hydrophobic F or W, has a cysteine residue, conserved across all three potato BSLs as well as the Arabidopsis orthologues. Nagano *et al.* (1999) show Cysteine to behave similarly to Tryptophan in structural analysis, and class it as strongly hydrophobic, so it may be that this KKIIC motif in the BSLs can indeed function in binding to a phosphatase in the same manner as an RVxF motif. In addition, cysteine residues can be subject to post-translational modification, for example by fatty S-acylation, which could also contribute to hydrophobicity. Ongoing work has confirmed this family of StBSLs to be S-acylated *in planta* within the C-terminal half of the protein, but the specific cysteine residues at which this occurs remain to be identified.

During preparation of this manuscript, it has been discovered that two amino acid motifs (KKLV and LKIKG) at the C-terminus of PiAVR2 facilitate the interaction between it and the BSLs (Frederic Brunner, personal communication). Further, work by Eleanor Gilroy has shown that a mutated AVR2 lacking either of these motifs cannot be recognised by R2, so co-expression does not result in an HR. Whether these AVR2 mutants are still functional in boosting pathogen virulence remains to be investigated. While these motifs do not conform to the classic RVxF motif, many more motifs have now been linked to PP1 binding (Heroes *et al.*, 2013; Cohen 2002), with interaction shown to occur at multiple regions between phosphatase and regulator. The binding regions of the mammalian PP1 inhibitor I-2 include the motifs ‘IKGI’ (Bollen, 2001) and

'KLHY' (Yang *et al.*, 2000), of which 'IKG' and 'HLK' can be found in the region of PiAVR2 shown to interact with the BSLs. Additionally, it should be considered that the PPKLs such as the BSLs are a different class of phosphatases from the well-characterised PP1. There are regions of conservation (see **Figure 6.1**), but with StBSL1 and human PP1 sharing only 43% identity, they may well have binding specificities that have not yet been characterised. The alignment with PP1 shows high conservation of the metal binding residues in the StBSLs, and also those residues involved in interaction at the hydrophobic groove. The RVxF docking sites show more variability, notably most diverged in StBSL2b (StBSL2).

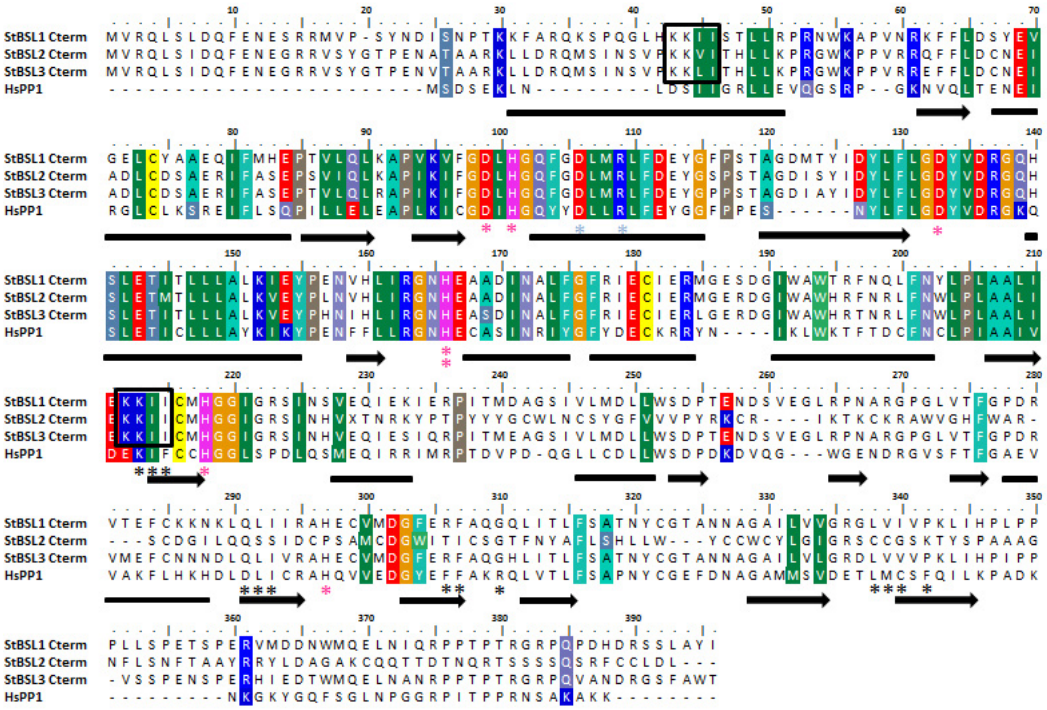

Figure 6.1 Amino acid alignment of StBSL family with mammalian PP1.

PP1C from *Homo sapiens* is shown aligned with the potato BSL family, with identical/similar residues back-coloured. Black boxes indicate the two motifs in the StBSLs that resemble KVxF motifs. PP1c topology is based on Peti *et al.* (2012). Alpha-helices are indicated by solid black bars, beta-strands by black arrows. Black asterisks indicate those residues important for forming the RVxF binding pocket. Blue asterisks indicate residues required for interaction of interactors/substrates at the hydrophobic groove. Pink asterisks indicate residues required for metal co-ordination, with the double asterisk indicating the histidine mutated to create phosphatase-dead StBSLs. Alignment generated using ClustalW in BioEdit.

It may be that PiAVR2 can bind the StBSLs in a regulatory manner, potentially influencing substrate specificity. Saunders *et al*. (2012) have shown that R2 only interacts with StBSL1 in the presence of AVR2. It is conceivable that PiAVR2 binds to a regulatory region of the StBSLs, allowing R2 to dock as a substrate. This is the case for the mammalian regulator MYPT1, which increases phosphatase activity towards myosin upon its binding to PP1, but decreases that towards glycogen phosphoryase (Bollen, 2001). PiAvr2 could also be viewed as an inhibitor in this context, as its binding could prevent other regulators or substrates (possibly the other BSLs) from docking. Another fascinating observation is that the non-recognised form of the effector, PiAVR2-like, has only one of these motifs intact, which could potentially be the key to its ability to evade R2-mediated recognition. If indeed R2 is a substrate of StBSL1, requiring dephosphorylation for downstream immune signalling, it may be that AVR2-like prevents this in some manner; perhaps by stearic hindrance of the substrate binding site of StBSL1, preventing R2 from docking. A pathogen effector functioning as a regulatory subunit of a phosphatase is not entirely novel; recently the effector Pi04314 was recently shown by Boevink *et al*. (2016) to interact with PP1c isoforms *in planta* by means of an RVxF motif. This results in relocalisation to the nucleus, found to be key to its function in attenuating JA and SA-response genes and boosting pathogen virulence.

Alternatively, these motifs in PiAVR2, and those in the StBSLs, could potentially be implicated in trafficking the proteins, or trafficking the complexes in which they exist. The C-terminal dilysine motif KKxxx, or KxKxx, has now been shown to facilitate localisation to the endoplasmic reticulum in plants (Benghezal *et al*., 2000) as well as

mammalian cells (Jackson *et al.,* 1990). While these have largely been identified at the extreme C-terminus of proteins, they may also be internal (Custer *et al*., 2013; Trujillo *et al*., 2010). Confocal microscopy was used to assess localisation of the PiAVR2 - StBSL1 interaction by Breen (2012). Fluorescence was largely considered to be cytoplasmic, and possibly plasma membrane. However the signal did correlate, albeit slightly more diffusely, with the ER when compared to a fluorescent ER marker. There may be distinct populations of these proteins in plants, localised to varying cellular regions depending on their activation state or interaction with other regulators. Localisation of PiAVR2 and the StBSL family should perhaps be revisited, particularly to compare wild-type to mutants lacking these motifs. In addition to confocal microscopy, they could also be examined using a cellular fractionation approach which would allow an assessment of the relative protein levels in each locale.

The discovery that these BSL family members do not have overlapping function adds an extra layer of complexity to their function as effector targets. Results in this work have shown that StBSL2b may play a largely opposite role to that of BSL1 and 2a; suppressing the AVR2-R2 HR as opposed to increasing it, and increasing INF1 cell death rather than suppressing, when transiently expressed in *N. benthamiana*. As discussed at the end of **Chapter Three**, this is supported by recent work by Maselli *et al.* (2014) who show AtBSL2b silencing to result in a phenotype bearing more similarity to BR-overactive mutants, suggesting that the protein is not a positive regulator of BR signalling as originally thought. This raises the question of which of these BSL family members PiAVR2 is actually targeting – why would the effector interact with all three, if they have opposing effects on immunity? This may again come down to an issue of

regulatory mechanism between family members, possibly disrupting the balance of homo vs. hetero-oligomers. An interesting point of note is the suppression of INF1 cell death by StBSL1 and StBSL2a, not just by the wild type versions of the protein but also the phosphatase-dead mutants. This implies that their phosphatase activity is not required for this effect. StBSL2b however, shows an increase in INF1 cell death with the WT protein, but suppression with the phosphatase-dead mutant, suggestive of a requirement for enzymatic activity. It may be that StBSL1 and 2a regulate BR signalling via protein-protein interaction rather than phosphatase activity – they may oligomerise with StBSL2b, disrupting the potential negative regulation of BR signalling by StBSL2b homo-oligomers, or behave as adaptors to alter the substrate specificity of StBSL2b. The interplay between these family members is more complex than originally thought, and understanding their regulation is a fascinating area for future work.

6.2 Susceptibility factors

Much of what is known about effector function centres on the suppression of the immune response, and more specifically; achieving this by down-regulation or inhibition of a host target protein. The alternate mode of effector function takes advantage of host susceptibility (S) factors, defined as 'plant genes that facilitate infection and support compatibility' (van Schie and Takken, 2014). Overexpression of an S factor would therefore result in increased virulence, silencing the gene would reduce virulence, or both may be true. While there are many examples of plant genes conducive to infection (for example, all endogenous negative regulators of host immunity can be considered S factors) there are only a few examples of S factors being directly targeted by pathogen effectors. Kay *et al*., (2007) describe the direct up-

regulation of the bHLH transcription factor Upa20 in pepper by the *Xanthomonas campestris* TAL effector AvrBs3. This results in increased cell size and is thought to play a role in pathogen nutrition. Another example from *Xanthomonas* are the multiple TAL effectors from *X. oryzae* which upregulate SWEET genes in rice – increasing sugar transport to the apoplast, again presumably for pathogen nutrition (Streubel *et al*., 2013).

This virulence strategy of targeting S factors has only recently been identified in filamentous pathogens. In addition to PiAVR2 targeting StBSL1 as detailed in this work, three other *P. infestans* effectors have recently been shown to increase pathogenicity by targeting host S factors in the host plant potato (reviewed by Boevink *et al., 2016*). These are Pi04314, which targets PP1c isoforms as already discussed (Boevink *et al.*, 2016), Pi04089, which targets the RNA binding protein StKRBP1 (Wang *et al*., 2015), and Pi02860 which targets the predicted E3 ligase StNRL1 (Yang *et al*., 2016). The role of S factors in enhancing filamentous pathogen virulence is represented in **Figure 6.2**.

These S factors represent an interesting focus for crop improvement – could specific examples be silenced in the plant, reducing susceptibility thereby increasing resistance? This may work in some cases but not others. The host protein in question generally does not exist solely to be a pathogen target (except in cases of decoy), and so it will have physiological purpose for the plant. Therefore silencing a susceptibility factor may have negative consequences, although depending on the context, a slight fitness cost may be worthwhile if it results in effective disease resistance.

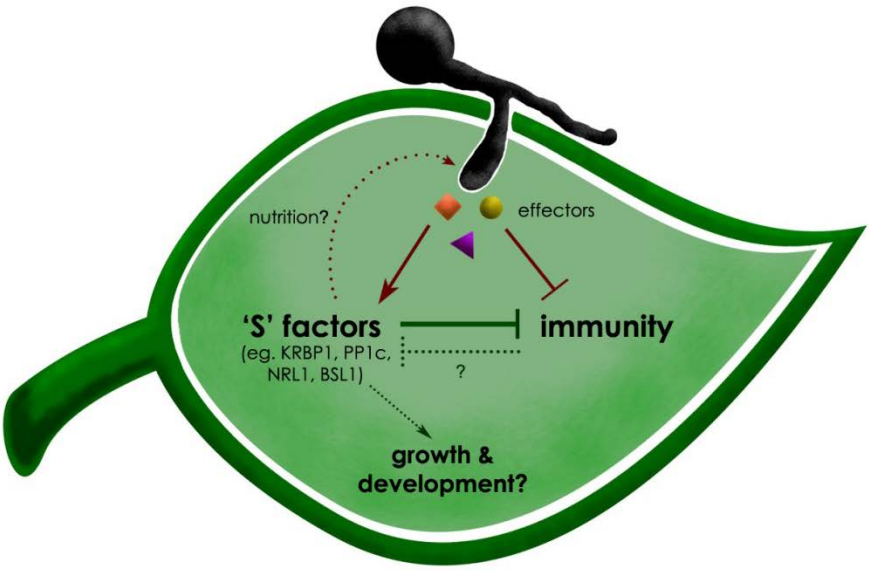

Figure 6.2 *P. infestans* exploits 'susceptibility factors' as effector protein targets

P. infestans has been shown to target a number of proteins in the host plant *S. tuberosum* which increase pathogen virulence when over-expressed. This increased virulence is due in some examples to antagonism of immunity (which may be reciprocal), or may be the result of improved pathogen nutrition. Adapted from Boevink *et al*. (2016).

For example removing a potent negative regulator of immunity could result in constitutive activation of immune signalling, which may provide increased (and possibly broad spectrum) disease resistance, but would likely be a significant drain on the plants resources and thus have a negative effect on growth and yield. One success story to come from using S factors in resistance breeding is mutation of the Mlo gene in barley, which codes for a transmembrane protein involved in the negative regulation of immunity. Mutation confers resistance to all races of powdery mildew by preventing pathogen penetration, and possibly impeding haustoria formation (Jorgensen, 1992). Side-effects, such as a slight reduction in grain yield and necrotic leaf spotting, were overcome by further breeding efforts and the mutant allele has remained durable since the 1980s. It has not been entirely without drawbacks however, and has recently been shown to convey increased susceptibility to the

emerging pathogen *Ramularia collo-cygni*, the causal agent of Ramularia leaf spot which is increasingly an issue in Europe and temperate regions (McGrann *et al.,* 2014). Van Schie and Takken (2014) describe the potential for increased durability of *S*-gene mediated as opposed to *R*-gene mediated resistance. Overcoming *R* gene-mediated resistance can be as simple as a single chance amino acid change in an effector, meaning the resistance gene no longer recognises it. On the other hand, overcoming *S*-gene mediated resistance would require the pathogen to overcome its dependency on that particular plant protein, and to achieve its virulence function in a completely different manner; ultimately more challenging for the pathogen to accomplish.

In the case of the StBSLs as susceptibility factors, silencing them does not appear to be a valid strategy for resistance. Silencing StBSL1 had no effect on pathogen growth, and while silencing StBSL2a, or StBSL2a and 2b, in *N. benthamiana* resulted in the strong suppression of disease symptoms, the dwarf phenotype and increased cell death phenotype of these plants would make cultivation difficult if not impossible. However, modern biotechnological approaches enable not only the addition or silencing of a gene, but also control over levels of expression. A genome editing approach such as Crispr/Cas9 technology, which utilises RNA-guided nucleases, may prove useful in this context; allowing the targeted alteration of a gene by point mutation, insertion, deletion, or altering transcriptional regulation (Bortesi and Fischer, 2015). This approach can introduce genome modifications that are indistinguishable from those introduced by traditional breeding or mutation techniques, thus may be considered non-GM by regulatory bodies (Belhaj *et al*., 2013). It may be that subtle adjustment of the expression levels of the individual BSLs relative to each other may be beneficial, but further research into the relationship between them is required.

6.3 Brassinosteroids and crop breeding

Brassinosteroids and the brassinosteroid signalling pathway have long been a point of focus in crop development, with both increased and decreased BR outputs proving useful. Che *et al*. (2015) show that *GL2*, an allele of the rice gene *OsGFF4*, confers increased sensitivity to brassinosteroids and increased BR signalling outputs, resulting in enlarged grains and a 16.6% increase in yield. This allele contains mutations that relieve its negative regulation at the transcriptional level, whilst leaving negative regulation at the protein level intact, resulting in only a modest increase in protein levels. In contrast, Dockter *et al*., (2014) describe the mapping of a range of barley mutants with reduced BR biosynthesis, resulting in resistance to lodging due to their shorter culms; a beneficial trait in high-yielding varieties which can be top-heavy. Gibberellin mutants in cereal crops can also be used for this same purpose, but BR mutants hold the advantage, with a more upright growth habit and erect leaves. This allows denser planting, and has been shown to result in increased yield per area in rice (Sakamoto *et al*., 2006).

The reciprocal antagonistic crosstalk between brassinosteroid signalling and the immune response identified in plants highlights a potential limitation to breeding efforts - will pushing for increased growth and higher yields lead to weaker plant defences? There may be ways to uncouple the two processes, which may work when resources are not limited, but could prove detrimental otherwise. A negative regulator of immunity, for example the transcription factor StHBI1-like identified in this work, could be knocked down or reduced in its expression, to relieve excessive immune suppression. The reverse scenario is also an issue – pushing immunity can inhibit

growth and thus have a negative effect on yield, so crosstalk in both directions would require fine-tuning for this to be feasible.

6.4 Pathogen manipulation of hormone pathways

Manipulation of phytohormone pathways by pathogens is a well-used virulence strategy, with pathogens benefitting from this in two main ways; by altering the plant's physiology, or by altering the plant's immune response. The major immune-regulating hormones SA and JA are apparent as the most frequently exploited, but pathogens have also been shown to manipulate ethylene, gibberellic acid, cytokinin, auxin, and abscisic acid signalling (reviewed by Kazan and Lyons, 2014). Effectors may mimic plant hormones to achieve this, exemplified by the JA mimic coronatine produced by *P. syringae* (Geng *et al*., 2014). This results in increased JA signalling, exploiting the antagonistic cross-talk between JA and SA signalling (Glazebrook, 2005). In terms of altering plant physiology, the root-colonising pathogen *Ralstonia solanacearum* has been shown to secrete auxins, increasing lateral root branching in the host plant petunia (Zolobowska and Van Gijsegum, 2006; Valls *et al*., 2006).

Whilst the brassinsteroid co-receptor BAK1 has been shown to be targeted by the effectors AvrPto, AvrPtoB, and HopF2 secreted by *P. syringae* pv. tomato DC3000, this is in the context of BAK1 as a co-receptor of PRRs such as FLS2, and thus the primary role of these effectors is to directly suppress immune signalling (Cheng *et al*., 2011; Shan *et al*., 2008, Zhou et al., 2014). Further, work by Xiang *et al*. (2011) suggests that FLS2 is the true target of AvrPto, and that its co-localisation and co-immunoprecipitation with BAK1 is indirectly by association. The up-regulation of the

BR pathway by PiAVR2 to indirectly antagonise immunity is therefore a novel virulence strategy in plant-pathogen interactions.

Cross-talk between biological systems, and its exploitation by pathogens serves as a reminder to keep the bigger picture in mind when considering plant microbe interactions. Whilst in depth knowledge of specific effector-target combinations is invaluable, in a 'real-life' situation a plant is subjected to a barrage of microbes; beneficial and parasitic, virulent and avirulent; and the responses that these interactions elicit in the plant must be carefully co-ordinated. The outcome of a particular plant-microbe interaction is dependent on the integration of immune, developmental and environmental signals, rather than one signalling pathway in isolation. Systems biology offers a means of modelling these interactions, utilising gene regulatory networks, protein interaction networks, co-expression modules and more, enabling the identification of key regulatory hubs open to exploitation by pathogens (Pritchard and Birch, 2011; McCormack *et al*., 2016; Mine *et al*., 2014). Progress in this field will allow us to consider a specific effector-target interaction in the context of the whole plant.

6.5 Breeding for resistance

As well as generating disease resistance by mutating susceptibility factors as already discussed, there are several other strategies that can be used in crop breeding efforts. While introgression of a single R gene does in theory have the potential to be easily overcome in the evolutionary arms race, in practice some R genes prove more durable than others, likely related to the essential nature of the particular effector protein

recognised (Jones *et al*., 2014). Whilst some effectors show redundancy and thus can be dispensed with, others are crucial to pathogen success, such as PiAVR3a (Bos *et al*., 2010), Pi03192 (McLellan *et al.*, 2013), and PiAVR2 (Breen, 2012). However in these cases, allelic diversity, where the effector function is maintained, but accumulates mutations that allow it to evade recognition, can also render R proteins useless. One success story of R protein breeding is the late blight resistance protein Rpi-blb2, introgressed into the Bionica and Toluca potato cultivars from the wild relative *Solanum bulbocastanum.* This has proved to be durable in the field thus far, but took 30 years of breeding effort to generate (Havervort *et al*., 2009). Another promising development in late blight resistance is the introduction of the resistance gene Rpi-vnt1.1 from *Solanum venturii* (Foster *et al*., 2009; Jones *et al*., 2014). This maintained 100% resistance to *P. infestans* in a three-year UK field trial, despite the prevalence of ideal blight conditions in its final year.

Building upon this, R gene stacking, where multiple resistance genes are deployed in the same cultivar, can be a more effective means of crop protection, offering more durability in the field. Jo *et al*. (2014) describe the stacking of two *P. infestans* resistance genes, Rpi-vnt1.1 and Rpi-sto1, from wild potato relatives into commercial varieties. This resulted in an increased spectrum of resistance, and was achieved using a marker-free approach, so no antibiotic resistance gene was incorporated into the genome. Additionally, these resistant plants can be considered 'cisgenic' as opposed to 'transgenic', as the resistance genes were introduced from crossable relatives. This method is considered comparable to conventionally bred plants in risk assessment

(EFSA, 2012) and as such may be a very useful concept in gaining public trust in biotechnology.

The issue of allele specific recognition, where a resistance protein recognises one effector variant but not the other, has driven the development of 'artificial evolution' techniques in R protein engineering. Regions of the gene are mutated, and screened for an enhanced or broader recognition spectrum. Chapman and Stevens *et al*. (2014) report an R3a variant mutated to gain recognition of the virulent *P. infestans* effector $Avr3a^{EM}$, in addition to the avirulent form $Avr3a^{KI}$. Unfortunately in this case the increased recognition spectrum did not translate into increased disease resistance.

This approach was based on the success of Farnham and Baulcombe (2006), who successfully mutated the potato NLR Rx, increasing its recognition spectrum to include additional potato virus X (PVX) strains as well as poplar mosaic virus (PopMV).

Both of these studies utilised error-prone PCR of the LRR domain; the region responsible for effector recognition. While increased activity of R proteins is desirable, this has to be maintained within fine levels to avoid over-activation, and immune signalling when no elicitor is present. This was the case in plants containing the broad-spectrum Rx mutant, which displayed a trailing necrosis phenotype when challenged with PopMV. This was rectified by mutagenesis of the nucleotide binding pocket at the N-terminus of the protein, the domain which dictates active/non-active signalling (Harris *et al*., 2013).

The concept of 'integrated decoys' represents a new, exciting angle from which to approach resistance engineering. This is based on the finding that many plant NLRs

have additional, highly variable domains that show similarity to proteins implicated in the immune response, such as Arabidopsis RRS1 which contains a WRKY domain (Cesari *et al*., 2014). The particular NLRs in which this has been identified are examples of resistance proteins which directly recognise their cognate effector protein, and it is hypothesised that these have evolved from part of the original virulence target being integrated into the NLR, thus acting as a decoy. RRS1 has now been shown to recognise the acetyltransferase effector PopP2 from *Ralstonia solanacearum*; an effector which acetylates WRKY transcription factors, resulting in the transcriptional suppression of multiple defence-related genes. PopP2 acetylates the decoy WRKY domain of RRS1, resulting in activation and immune signalling (Le Roux *et al*., 2015). This integrated decoy mechanism is thought to be a widespread occurrence in the plant NLR complement, with WRKY, BED zinc finger, and kinase domains most frequent (Kroj *et al*., 2016). This represents not only an elegant strategy for identifying effector targets based on similarity to these integrated domains, or 'IDs', but also offers the potential for developing synthetic resistance proteins with integrated decoys specific to common effector targets.

Monoculture is a point of serious weakness with regard to crop disease control. Large fields of genetically identical plants mean that if disease takes hold on one plant, it can easily infect the entire field. However the benefits of monoculture (largely the hugely increased efficiency that it affords) mean that this practice is difficult to get away from. How can we re-introduce variety into our fields, whilst maintaining efficiency in our agricultural systems? One solution is the development of multiline cultivars- bred to be phenotypically identical, differing only in their disease resistance (Mundt, 2002).

Cultivar mixtures are another option; these are cultivars with differing disease resistance, selected for similarity in agronomic traits but not identical. Zhu *et al*. (2000) report spectacular success with this technique in rice, with 93% decrease in rice blast, yield increase of 89%, and fungicide application reduced to zero by the end of the project.

As an alternative to R proteins, quantitative trait loci, or more specifically, quantitative resistance loci (QRL), also hold potential for improving disease resistance. In contrast to a single R gene which can provide full or qualitative resistance, QRLs contain multiple genes that each confer a more modest resistance. The exact mechanism for this is as yet unclear; they may be weak *R* genes, be involved in defence signal transduction, or detoxify pathogen compounds, among other hypotheses (Poland *et al*., 2009).

Genetic engineering offers enormous potential for improving the disease resistance of our crops – making it possible to introduce a beneficial gene or genes straight into established landraces, thus maintaining the favourable traits for which it was initially bred. The strategies for resistance discussed here will be largely impossible to implement without this technology. Resistance may be introgressed via more conventional means by selective breeding, but even for a single *R* gene this is time consuming, and requires multiple back-crosses to restore the original combination of favourable traits. Genetic engineering also offers more scope for subtlety in crop improvement; not only introducing a beneficial gene or genes, but also enabling control over expression levels and organ-specific localisation. Whilst other parts of the

world are making progress utilising this technology as a tool to improve agriculture, Europe is held back by government policies that largely prohibit the growing of genetically modified (GM) crops, with public perception remaining more negative than positive. A major factor in this prohibition is perhaps a lack of understanding. This is reflected in poll results from YouGov Plc in 2014 (https://yougov.co.uk/news/2014 /02/21/many-britain-remain-sceptical-gm-foods/). While 40% of respondents were decided against the technology, almost the same number (38%) of respondents selected the option 'don't know' when asked if the government should be promoting the adoption of GM in the UK. This suggests that a vast proportion of people don't feel they know enough about the topic to make a decision. It is imperative that science is better communicated to the general public, and to political powers, to enable informed decisions and to give the UK a fighting chance of competing in the global food market in the future. With evidence supporting the safety of such technology (Batista and Oliveira, 2009; Cockburn, 2002), and an overwhelmingly positive result for farmers (Carpenter, 2010), there appears to be no logical reason for a blanket ban on a tool with such potential.

Whether biotechnological approaches or more traditional breeding techniques are used to improve crop varieties, the durability of the resistance is dependent not only on the research and development that goes into creating the resistant variety in the first place, but also on good deployment and management of this resistance in the field. This requires a collaborative effort from all aspects of biology, from genetics and molecular biology, to social and environmental sciences (Mundt, 2014).

6.6 Future Work

The research described in this study succeeded in answering some of the questions surrounding the role of the effector PiAVR2 in *P. infestans* virulence. However it also raises many more, and has led to many ideas for future study, presented below.

1. How is the AVR2-BSL interaction monitored by R2? The discovery that phosphatase activity of BSL1 is necessary for the HR suggests the possibility that R2 may be dephosphorylated by BSL1 to initiate immune signalling. Tagged R2 could be immunoprecipitated and tested by Western blot probed with phospho-specific antibodies.
2. What is the function of the individual BSLs in brassinosteroid signalling? Whilst work has shown that PiAVR2 binds with all three family members, and that PiAVR2 increases brassinosteroid signalling, what is lacking is an assessment of each BSL proteins contribution to this. Developing a set of single, double and triple knock-outs in either *N. benthamiana* or potato itself would be ideal, and would build upon the data gained from virus-induced gene silencing. BR-marker gene expression could be assessed both with and without BR treatment.
3. What are the substrates of the StBSL proteins, apart from the other family members? Given the published interaction between AtBSU1 and AtBIN2, identifying a BIN2 orthologue in potato and testing by co-immunoprecipitation would be a good starting point. A potential potato BIN2 orthologue had been previously examined in yeast2-hybrid analysis, but unfortunately proved to be auto-active in this system. To widen the search, Yeast-2-hybrid library screening would be useful to identify potential candidate interactors of the BSLs to take forward. Alternatively, BSL proteins could be immunoprecipitated and the

sample analysed using mass spectrometry, to identify proteins which co-immunoprecipitate. Identifying substrates would allow investigation of the regulation between BSL family members, as their phosphorylation state could be assessed in the presence or absence of different family members.

4. What is the function of the kelch domain in the StBSLs? Work in this study focused on full length and phosphatase domain constructs of the StBSLs, and the results from several experiments made it clear that the phosphatase domain did not have the full function of the intact protein. It may be that the kelch domain has entirely independent function, or alternatively the combination of kelch and phosphatase may be required, to facilitate protein-protein interactions that contribute to phosphatase activity. Ideally the remaining N-terminal fragment of the genes would be cloned, and tested for effect on the AVR2-R2 HR, INF1 cell death, and *P. infestans* leaf colonisation.
5. Protein modelling – it would be extremely informative to have crystal structures for PiAVR2, the StBSL family, and SdR2, to enable modelling of the interaction between effector, target and resistance protein.
6. S-acylation of StBSLs and SdR2 – this post-translational addition of a fatty acid group to proteins can be associated with localisation, trafficking, protein activity and interactions, and is unusual in being the only lipid modification to be reversible. All three StBSLs as well as SdR2 have now been confirmed as being S-acylated, and it would be interesting to investigate what role this plays. Is it simply a matter of tethering these proteins to the required localisation, or does their S-acylation have a more complex regulatory role?

6.7 Concluding Remarks

It is likely to be the case that there will be no 'cure-all' solution to the problem of potato late blight, and that the battle will be an ongoing one. The pathogen *Phytophthora infestans* has proved remarkably adaptable and resilient, quickly overcoming much of the resistance deployed in the field, as well as evolving insensitivity to some pesticides. However it can be hoped that improvements in our understanding of plant-pathogen interactions at the molecular level will enable us to devise more and more durable strategies for resistance in the future, not only to *P. infestans* but to other challenging crop diseases that threaten global food supply.

The work detailed in this study has impact first and foremost on the understanding of pathogen effectors and their function in virulence; a thriving field in plant science. In addition, the particular example of PiAVR2 and its interaction with the BSL family has proved informative in beginning to understand the crosstalk between the brassinosteroid pathway and immune signalling in the crop plant potato. Manipulating this crosstalk to suppress host plant immunity is a sophisticated and effective means of increasing pathogenicity, and could prove relevant to many other plant-microbe interactions. Beyond this, the goal of research in the field of plant pathology is to inform crop breeding strategies of the future, in the hope of contributing to increased disease resistance, decreased use of pesticides, and ultimately sustainable food security. The world population is predicted to surpass 9 billion by 2050, and while crop yields are still growing, the rate of this growth is beginning to decline (Alexandratos and Bruinsma, 2012). Thus we must seek improved, intelligent ways of increasing food

production, whilst minimising the depletion of resources, and maintaining balance with the environment.

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